STUDIES ON ENZYME DISTRIBUTION IN SUBCELLULAR PARTICLES OF LIVER AND THYROID

by 45

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INTRODUCTION

Investigations of subcellular enzyme systems have up to the present time been hampered by an inability to cleanly separate subcellular components. The vast majority of this work has concentrated on subcellular enzyme systems of rat liver, the result being a tendency to accept all data obtained from this organ as being a model for all other tissues and their enzyme systems. Little attention has been paid to the possibility that other tissues may exhibit different subcellular properties and enzyme distributions.

The study was therefore undertaken to determine if a species difference did exist and if so how it was manifest in the enzyme distribution as exhibited on a density gradient. To this end a fractionation scheme was employed for the separation of rabbit liver mitochondria fraction subcellular components. This work describes the separation of subcellular particles on a ficoll density gradient and compares it to classical work done on a sucrose density gradient.

In dealing with tissues other than liver, it was observed that subcellular work on organs of the endocrine system was scarce. A fractionation scheme was therefore set up for the thyroid gland, whose main function is seen as the regulation of metabolic rate.

Of particular interest in working with this gland was the possibility of elucidating something of the action of thyroid peroxidase. Iodination as well as peroxidation properties have recently been ascribed to this enzyme. This work has thus suggested a prominent role for thyroid peroxidase in the biosynthesis of thyroxine, the hormone elaborated and secreted by the thyroid for the regulation of metabolic rate.

Numerous separation and purification schemes have been employed by various authors for this enzyme. All of these experiments have pointed to the fact that nothing is known of the exact locus of action of the enzyme. In recent years evidence has suggested that there may be more than one peroxidase in the thyroid. This evidence, however, has been based on imperfect separation schemes with little or no knowledge as to which subcellular particles were involved.

This thesis describes the separation and identification of thyroid subcellular particles with special emphasis on the location and characterization of peroxidase. It also describes the studies made to determine if more than one peroxidase is involved in thyroid metabolism.

REVIEW OF THE LITERATURE

I. Historical Evolution of Liver Fractionation Methods and Enzyme Distribution

A. The Discovery and Isolation of Lysosomes

Impetus to the field of particle fractionation was given by the discovery that a group of acid hydrolytic enzymes was concentrated in the light mitochondrial fraction isolated from liver homogenates of rats (de Duve, et al. 1955). This earlier work was achieved by employing the technique of differential centrifugation. Data were later collected on the enzyme distribution of the mitochondrial fraction of rat liver through density gradient centrifugation (Beaufay, et al. 1959). The density gradient studies of Sawant, et al. (1964) confirmed the findings of these workers that all populations of lysosomes were confined to the light mitochondrial fraction of rat liver. The results obtained in these investigations provided confirmation of the existence of lysosomes as a separate group of particles, distinct from mitochondria.

1. Properties of Lysosomes

In view of the changing properties of lysosomes during their life cycle, a brief outline of lysosomal terminology suggested at a symposia on lysosomes (deReuck and Cameron, 1963) is presented at this point.

> Primary lysosome: a cytoplasmic granule showing high concentrations of several acid hydrolytic enzymes which are not now and have not before been functional.
>
> Secondary lysosome: a cytoplasmic granule whose acid hydrolytic enzymes are now or have been functional.
>
> Phagolysosome: a body functioning in the digestion of exogenous cell material which has been incorporated into the cell by the process of phagocytosis or pinocytosis.
>
> Autolysosome: a body functioning in the digestion of endogenous cell material.

The importance of the lysosomal membrane for the activation of the hydrolytic enzymes in vitro and in vivo was recognized by de Duve and co-workers early in their work. These investigators systematically studied various treatments which injured the membrane, thus releasing the enzymes. These treatments included osmotic shock, freezing and thawing and incubation at 37° C (deReuck and Cameron, 1963). Pangborn and Tappel (1965) observed that the enzymes were released from kidney lysosomes in two stages during incubation at 37° C. In the first stage, increasing amounts of enzymes became available to the external substrates but the granules did not release their enzymes and did not show any change in morphology. In the second stage the enzymes were released at the same time as the granules disintegrated. The enzyme activation during the first stage still remains obscure. Reports in the literature also indicate that the properties of lysosomal membranes are not uniform and are varied with their physiological state. However, the factors which cause the release of enzymes from lysosomes are still little understood.

2. Complete Resolution from Mitochondria and Peroxisomes

It was found by Wattiaux, et al. (1963) that injections of suitable doses of the neutral detergent Triton WR 1339 produced massive swelling of the lysosomes with a concommitant decrease in their density, but was without effect on other subcellular particles. By this means it was possible to obtain a reasonably pure preparation of these particles (Wattiaux, et al. 1964 and Baudhuin, et al. 1965).

Beaufay, et al. had noted in working with glycogen gradients that the inclusion into the gradient of 5% of the substance ficoll, introduced by Molter and Moller (1958), caused a slight increase in the equilibrium density of cytochrome oxidase, total protein and catalase, but not of acid

phosphatase. These same workers had also worked with a linear ficoll gradient which contained a constant concentration of sucrose throughout, but had found all enzymes and protein to be concentrated in one narrow band. Thus, the use of Triton WR 1339 to cause a selective decrease in lysosomal density through phagocytosis of the detergent by these particles appeared to be the only way in which the lysosomes could effectively be separated from other particles and was used in all subsequent analysis of subcellular particles (Baudhuin and de Duve, 1966 and Leighton, et al. 1968).

B. The Discovery and Isolation of Peroxisomes

The term "microbody" was introduced into the literature in 1954 to designate a special type of cytoplasmic body present in mouse kidney cells. This body was characterized by a single membrane and a fine granular matrix. Two years later, particles resembling kidney microbodies but containing in addition a dense core with a regular crystalline structure were described under the same name in the cells of rat liver by Rouiller and coworkers (1956), who suggested that these particles were precursors of mitochondria.

Biochemical experiments which eventually led to the characterization of microbodies were initiated in 1953 as a consequence of the finding by Novikoff that in rat liver the enzyme uricase showed sedimentation properties similar to those of acid phosphatase. It was at this time that lysosomes were being studied and characterized by the Louvain Laboratory through similar studies on acid hydrolase (de Duve, et al. 1953 and de Duve, et al. 1955). Urate oxidase was added to the list of enzymes under study and it was found that this enzyme did not display the solubility and latency properties characteristic of the lysosomal hydrolases. It was concluded at this time that urate oxidase was attached "either to the insoluble framework of lysosomes or to a fourth distinct group of granules with the properties of

large microsomes." (de Duve, et al. 1955) This ambiguity was resolved in 1963 by the finding that Triton WR 1339 selectively decreased the density of lysosomes and left the uricase containing bodies unchanged. Using glycogen as the solute in density gradient analysis in the presence of low concentrations of sucrose also made it obvious that urate oxidase was not a constituent of lysosomes (Beaufay, et al. 1964). Catalase and D-amino acid oxidase were found to have sedimentation properties similar to that of urate oxidase. It was also found that the microbodies of rat liver contain an L-alpha-hydroxy acid oxidase and that particles with similar biochemical properties exist in rat kidney and Tetrahymena pyriformis (Baudhuin, et al. 1965b).

These findings strengthened the hypothesis that the microbodies are important sites of hydrogen peroxide metabolism and that the association of the oxidases with catalase is biologically meaningful. In 1965 (b), de Duve put forth the biochemical term peroxisome to characterize this association, the term microbody thus being reserved for those particles which have been morphologically characterized only.

1. The Dense Uricase Containing Core

It was concluded from combined morphological and biochemical data that the crystalline core of the microbody may have a different substructure in different species and that this core may represent the site of urate oxidase activity (Baudhuin, et al. 1965a; Shnitka, 1966 and Tsukada, et al. 1966). Hruban and Swift (1964) found structures closely resembling isolated cores in a commercial preparation of urate oxidase and concluded along with Baudhuin and his associates (1965a) that the insoluble urate oxidase was probably associated with this core while the soluble catalase and D-amino acid oxidase are present in the structureless sap in which the core is embedded. It was pointed out by Baudhuin (1965a) that catalase and

D-amino acid oxidase appeared to exhibit a certain degree of latency and that depending on the degree of damage sustained by the particles in the course of isolation, free cores may be anything from virtually absent to representing the main morphologically identifiable component in sediments separated by density gradients. Sediments which were rich in isolated cores also showed an excess of urate oxidase activity over catalase and D-amino acid oxidase activities. De Duve and Baudhuin (1966), Leighton, et al. (1968) confirmed the fact that isolated cores of peroxisomes equilibrated at a higher density than intact particles.

2. The Complex Catalase Distribution

In 1954, Paigen postulated catalase to be a mitochondrial enzyme. This view was brought into question by Thomson and Klipfel (1957), who showed that catalase sedimented closely with urate oxidase in homogenates of mouse liver centrifuged through a stabilizing density gradient.

In view of the finding that particulate bound catalase activity was enhanced by treatment with Triton X-100 (Adams and Burgess, 1957), Feinstein (1959) put forward the hypothesis that this enzyme could be a component of the lysosomes which exhibited similar behavior. However, pretreatment of the animals with Triton WR 1339 to cause selective decrease in lysosomal density appeared to rule out catalase as a lysosomal component (Wattiaux, et al. 1964 and Baudhuin, et al. 1965).

Catalase and D-amino acid oxidase were both found to lose part of their activities when exposed to high sucrose concentrations. These enzymes were apparently released in a selective fashion from the dense particles and it was postulated that these particles had a large sucrose space unlike the lysosomes which behaved as osmometers (de Duve, et al. 1960 and Beaufay, et al. 1964). These experiments at the same time appeared to clear up the

misunderstanding that catalase was a mitochondrial enzyme (Paigen, 1954). This enzyme latency of catalase had also been noted by Vaes (1965) who noted that it was released by Triton X-100 but at a slower rate than were the lysosomal enzymes and that this latency was completely insensitive to media of low osmotic pressure. He therefore concluded that the catalase containing particles were probably different from the lysosomes (Vaes and Jacques, 1965).

The latency of catalase has made it possible to study the sensitivity of the peroxisomal membrane to various disruptive treatment. Of particular interest was the finding that approximately ten times more detergent was necessary to accomplish the same degree of catalase release as was necessary to bring about a release of acid phosphatase from the lysosomes (de Duve, 1965a). This difference indicated an inherent difference in the chemical composition of the two membranes and provided additional evidence for their lack of kinship.

II. Particulate Thyroid Fractionation and Enzyme Distribution

A. Peroxidase Purification

Various attempts have been made to isolate and purify thyroid peroxidase. Hosoya and Ui (1961) first characterized hog thyroid peroxidase as a particulate enzyme sedimenting between 5,000 x *g* and 80,000 x *g*. The following year, Degroot and Davis (1962) reported on the purification of a sheep thyroid peroxidase sedimented between 700 x *g* and 85,000 x *g*. This same year Klebanoff, *et al*. (1962) attempted a purification of calf thyroid peroxidase from 105,000 x *g* pellets. In 1964, Igo and coworkers worked out a purification procedure for peroxidase isolated from beef thyroid particles sedimented between 1,000 x *g* and 90,000 x *g*. This procedure was similar to that employed by Hosoya, *et al*. in 1962. Maloof and Soodak (1964) contributed

greatly to the work in this field by observing that hog thyroid peroxidase isolated from 8,500 x g to 105,000 x g particles was greatly stabilized by the addition of low concentrations of iodide and thiocyanate. In 1967, Coval and Taurog reported on the hog thyroid peroxidase isolated from 12,000 x g particles which was shown to be only slightly heterogeneous by ultracentrifugation.

During this period of experimentation, various authors obtained data which indicated the possibility of more than one peroxidase in the thyroid. Alexander and Corcoran (1962 and 1965) found an incomplete fragmentation which occurred during dissociation of the enzyme into heat labile apoenzymes and the hematin prosthetic group. In 1964 and 1965 Yip reported on a beef thyroid peroxidase obtained from subcellular particles which was separable into several fractions; one which oxidized $NADH_2$ and one which oxidized both $NADH_2$ and o-dianisidine.

B. Subcellular Localization of Thyroid Peroxidase

The varied centrifugation schemes employed by workers for the isolation of thyroid peroxidase made it obvious that the precise location in the subcellular particles is controversial. In 1967, Nicholson attempted to localize the peroxidase containing particles by density gradient centrifugation. This was performed on a 7-35% linear ficoll gradient. Peroxidase activity was associated with definite protein banding. However, assays for other subcellular components believed to be associated with the "mitochondrial fraction" were not performed so that it was impossible to specify any one particle as being the site of peroxidase activity. It was assumed by the author that this enzyme was probably associated with the true mitochondria since its activity lay in direct association with protein distribution.

C. Characterization of Lysosomal Components

As late as 1963, Hosoya attempted a separation of hog thyroid mitochondrial fraction according to the earlier techniques employed by de Duve (1955). He concluded from this work that acid phosphatase activity appeared to be concentrated in the lysosomal portion of the particulate fraction. Peroxidase activity, on the other hand, was found in heavy contamination in the majority of those fractions which had been separated by differential centrifugation. Although there was no real evidence as to the exact site of location of the enzyme, he postulated on the basis that the enzyme could be solubilized by digitonin that the membranes of the endoplasmic reticulum might represent the actual site of peroxidase localization (Hosoya, et al. 1962). Lysosomes were ruled out as the site of peroxidase location on the basis of the fact that the enzyme operates at a pH optimum of 7.8 whereas the lysosomal enzymes operate at acidic pH values. Herveg, et al. (1966) demonstrated acid phosphatase, beta-glucuronidase and cathepsin activity in calf thyroid homogenates and concluded on the basis that a latency existed for these enzymes that they were contained within lysosomes.

III. Synthesis of Thyroid Hormones and Relation to Subcellular Fractions

A. Microsomal Biosynthesis of Thyroglobulin-Bound Thyroxine

After many years of research, little direct evidence has been obtained to elucidate the mechanism of thyroglobulin synthesis. In 1964, the problem of localizing the site of synthesis of the protein moiety of thyroglobulin was attacked by Nadler and coworkers. Using tritium labelled leucine and radioautography, he followed the labelling of protein with time after injection and concluded that thyroglobulin formation proceeded through the following network of subcellular structures; protein formation on the ribosomes outside the walls of the cisternae, migration of the protein into

the lumen of the cisternae, movement to the Golgi zone for the probable addition of the carbohydrate complex and finally deposition of the glycoprotein in the apical vesicles. Work done by Cavelieri and Searle (1967) gave further evidence as to the particulate mechanism of thyroglobulin synthesis in vivo. By applying thyroid homogenates to a density gradient and measuring the C^{14} and protein content of each fraction as a function of time after injection of C^{14}-leucine, the following scheme was proposed for thyroglobulin biosynthesis:

a) formation of a 12s subunit, probably from other smaller polypeptide units;

b) aggregation of two 12s subunits into a 16s molecule;

c) and maturation of the 16s protein into 19s thyroglobulin.

B. Microsomal Iodination of Tyrosine Units

Nunez and coworkers (1965) showed that chemical iodination of amino acid labelled thyroglobulin derived from sheep thyroid slices resulted in an increase in sedimentation from 16s to 19s. Therefore, the 16s protein appeared to consist only of uniodinated thyroglobulin.

The question arose however as to the site of iodination in the cell. Mitmaker (1960) strongly contended that this reaction takes place only after reaching the lumen of the follicle. Evidence obtained by Nunez and coworkers (1967) indicated that this reaction is membrane bound and does not occur in the colloid. Ekholm and Stranberg (1968) indicated that thyroglobulin was iodinated before reaching the follicle lumen and that a transfer of protein from one particle--probably the ribosome-studded cisternae of the endoplasmic reticulum--to a granular vesicle surrounded by a unit membrane took place. This latter microsomal subfraction contained only 19s protein three hours after administration of H^3-leucine. However, the ribosome studded

cisternae also contained a 19s protein in as little time as thirty minutes after H^3-leucine injection, indicating that iodination had also taken place in this area. These workers had previously found that the specific activity of microsomal 19s thyroglobulin was higher than that of the supernatant 19s at 1 and 3 hours after H^3-leucine administration but decreased rapidly between 3 and 6 hours, simultaneous with the increase of supernatant 19s radioactivity, possible indicating a release of iodinated thyroglobulin into the colloid (Ekholm and Strandberg, 1967). Two years previous to this work, Degroot had solubilized an iodide peroxidase-tyrosine iodinase from mitochondria and microsomes of calf thyroid by treatment with deoxycholate, again suggesting that iodination is not an intercolloidal process.

C. Release of Active Hormone by Lysosomal Action

The role of acid hydrolases--cathepsin--in the degradation of colloidal thyroglobulin has been demonstrated by Wollman and coworkers (1964) and Nadler, et al. (1962). Their studies suggested that the initial event in the degradation involved the formation of small vesicles containing thyroglobulin which arise at the apical margin of the cell by a process of phagocytosis. At this stage, the vesicles stained with periodic acid Schiff reagent but acid phosphatase activity was not demonstrated. Vesicles were later seen which stained for both colloid material and acid phosphatase and it was considered that the enzymes were acquired by transfer from lysosomes originally present in the follicular cell region. As the vesicles moved toward the base of the cell, the periodic acid Schiff reaction became progressively weaker and it was surmized that during this time there was a degradation of thyroglobulin with formation of iodothyronines. This work was supported by Roitt who demonstrated a pH 3.5 acid phosphatase activity in human follicle cells and showed that this activity was absent from the col-

loid itself (Cassano and Andreoli, 1965). Herveg, et al. (1966) demonstrated acid phosphatase, beta-glucuronidase and cathepsin activity in calf thyroids which had been washed free of colloid material. These enzymes displayed a latency which was unmasked by Triton X-100 and dilution in hypoosmotic media. This is a characteristic reaction of lysosomal bound enzymes to such treatment.

IV. Microscopic Analysis of Subcellular Particles

A. The Classical Liver Model

1. Lysosomes

Progress during the early phase of lysosomal research was based mainly on the biochemical analysis of isolated fractions. In recent years, however, cytochemical methods and electron microscopy have contributed much to the advance of knowledge in this field. The identification of polymorphous bodies with pericanicular bodies as lysosomes (Novikoff, et al. (1956) was much facilitated by the application of the cytochemical acid phosphatase method of Gomori (1952). This tentative identification was confirmed by application of the Gomori procedure at the electron microscope by Holt and Hicks (1961), Novikoff (1963) and Miller and Palade (1964). Lysosomes were also localized by taking advantage of their ability to concentrate injected foreign protein. After injection of horseradish peroxidase or egg white, the size of many lysosomes increased, and the foreign proteins were found to be concentrated together with the hydrolytic enzymes in the isolated lysosome fractions (Strauss and Oliver, 1955 and Straus, 1957,a,b).

2. Mitochondria

It is known that many of the agents and treatments which cause lysosomal breakdown also result in mitochondrial disruption, e.g. incubation of purified particles at 37° C. Whittaker (1966) was able to

demonstrate that the elongated form of liver mitochondria was retained in fractions isolated in 0.44 M sucrose, but not in 0.25 M sucrose. Crofts and Chappell (1965) demonstrated that if a suitable chelating agent such as EDTA is added to the isolated media, swelling of the mitochondria by the presence of calcium may be prevented. If swelling was not prevented mitochondrial inclusions which have a characteristic hollow appearance were released.

3. Peroxisomes

Most of the electron microscopic analysis has been performed on liver and kidney peroxisomes. Up to this point, staining techniques have not been applied to these particles. Electron microscopy has revealed that the only unique feature of these particles appears to be the urate oxidase containing core which has a different substructure in different species (Baudhuin, et al. 1965; Shnitka, 1966 and Tsukada, et al. 1966).

B. Thyroid Subcellular Particles

It is well accepted that the active hormone of the thyroid gland, thyroxine, resides in an inactive form in the matrix of thyroglobulin (Pincus, et al. 1964). This glycoprotein must be degraded somehow into biologically active fragments. Wollman, et al. (1964) and Nadler, et al. (1962) processed electron micrographs showing the ingestion of colloid material containing thyroglobulin from the lumen into the thyroid cell. These droplets accumulated in the apical portion of the cell. Characteristic changes in the granules observed through cytochemical stains for acid phosphatase and esterase suggested a fusion of the droplets with lysosomes. Wetzel, et al. (1965) investigated the relationship between colloid droplets and lysosomes in the thyroid with the electron microscope and came to the conclusion that thyroxine was split from thyroglobulin within the phagolysosomes by the action of the lysosomal enzymes. This work provided one of the first examples of a biological

function of lysosomes.

EXPERIMENTAL PROCEDURES

I. Materials

A. Reagents

All reagents were prepared in deionized water which was obtained by filtering distilled water through a Barnstead Model BD-5 demineralizer. Ficoll was obtained from Pharmacia Fine Chemicals, Inc. Sucrose, paraformaldehyde, 30% hydrogen peroxide and succinic acid were purchased from Fisher Scientific Company. Bovine serum albumin (2X crystallized) was obtained from Armour Pharmaceutical Co. Calf thymus DNA was purchased from Worthington Biochemical Corporation. Para-nitrophenylphosphate, horse heart cytochrome c (type III), L-cysteine sulfinic acid, uric acid, thyroglobulin, NADH and 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) were obtained from Sigma Chemical Company. Guaiacol was obtained from Matheson Chemical Company and was redistilled before use. Phenazine methosulfate and FAD were obtained from Nutritional Biochemical Corporation. Dow Epoxy Resin 732 and 332 for electron microscopic analysis were from the Dow Chemical Company.

B. Liver Tissue

Laboratory rabbits were used as a source of this tissue. Males and females were used and no special dietary procedure was used. Water and food were provided ad libitum. The animals were sacrificed by a sharp blow to the occipital-atlas junction in order to effect instantaneous death. The livers were quickly removed, washed free of blood and put through the homogenization process as quickly as possible to avoid breakdown of subcellular particles.

C. Thyroid Tissue

1. Frozen

Most of the hog dry ice packed thyroid glands were obtained commercially from Swift and Co., Kansas City, Kansas. Others were collected at the Wilson Co. slaughter house in Kansas City and brought to the laboratory on ice. All thyroids were frozen at -20° C. to preserve enzymatic activity.

2. Fresh

This tissue was obtained from hogs at the Kansas State University slaughter house. They were collected at the time of slaughter, brought to the laboratory on ice, trimmed free of fat and used immediately.

D. Homogenization Medium

The homogenization medium for the isolation of subcellular particles from liver was prepared by dissolving 85.5 g of sucrose and 0.372 g of ethylenediaminetetracetic acid, disodium salt, in one liter of deionized water. This made a 0.25 M sucrose-1.0 mM EDTA solution.

The homogenization medium used for thyroid glands was prepared by dissolving 131.67 g of sucrose and 0.186 g of ethylenediaminetetracetic acid, disodium salt, in 500 ml of 0.1 M potassium phosphate buffer, pH 8.0 which contained 0.5 ml of 0.1 M KI. The final concentration of the solution was therefore 0.77 M sucrose-1.0 mM EDTA-0.1 mM KI in 0.1 M potassium phosphate buffer, pH 8.0. The concentration of sucrose was raised to 0.77 M in order to avoid osmotic rupture of the lysosomes. KI was added because of its known protective effect on peroxidase activity.

II. Methods

A. Preparation of Complete Mitochondrial Fraction from Liver

A rabbit liver was put through a Hobart tissue grinder with a

stainless steel cutting blade and 3/16 inch hole plate. The ground liver was then homogenized in three volumes of homogenization medium. This was accomplished with a smooth glass homogenizer fitted with a teflon pestle using a medium speed of rotation and only one stroke up and down to avoid undue breakage of mitochondria and lysosomes. The crude homogenate was centrifuged at 1,000 x g for 10 minutes to remove cell debris and nuclei. The supernatant was carefully separated from the sediment and centrifuged at 27,000 x g for one hour to obtain the complete mitochondrial fraction. The supernatant was discarded, the sediment washed once with a small amount of homogenization medium and recentrifuged at 27,000 x g for 1 hour. When a ficoll density gradient was employed, the mitochondrial fraction was washed with 20% ficoll (w/w). The resulting mitochondrial fraction was used in further density gradient analysis. A diagram of this procedure is presented in Figure 1.

B. Preparation of Complete Mitochondrial Fraction from Thyroid

Hog thyroids were removed of connective tissue by squeezing them through several layers of stainless steel mesh wires with the aid of a Carver tissue press. The pressed hog thyroid tissue was next homogenized with three volumes of homogenization medium using a smooth glass homogenizer fitted with a teflon pestle at medium speed and using one stroke up and down. Cell debris, lipid and nuclei were removed at 1,000 x g for 10 minutes. The resulting supernatant was centrifuged at 39,000 x g for one hour. The complete mitochondrial fraction was washed once with 0.1 M potassium phosphate buffered 10% ficoll-0.1 mM KI and recentrifuged at 39,000 x g for 20 minutes. The resulting mitochondrial fraction was used for density gradient analysis. A diagram of this procedure is presented in Figure 2. When the complete mitochondrial fraction was not employed in density gradient analysis the ficoll wash was deleted.

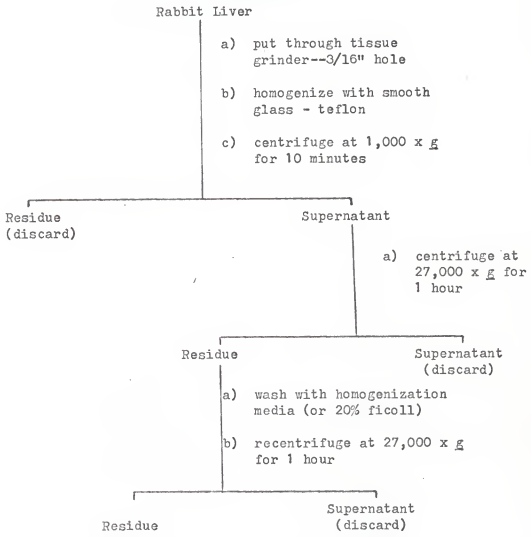

Fig. 1. Procedure for obtaining complete mitochondrial fraction from liver tissue.

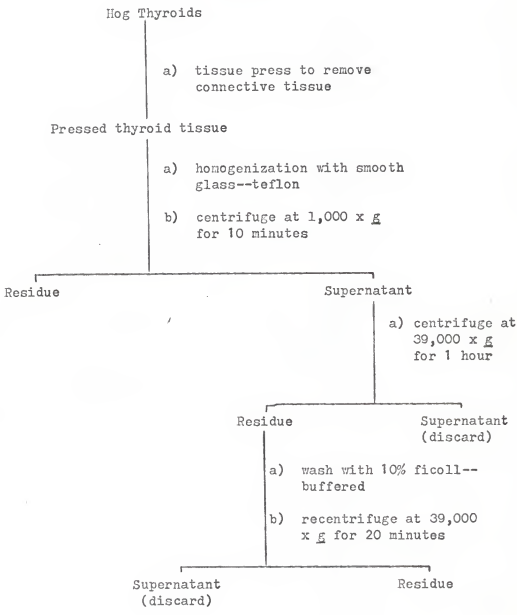

Fig. 2. Procedure for obtaining complete mitochondrial fraction from thyroid tissue.

C. Density Gradient Centrifugation of Liver Mitochondrial Fraction

1. Preparation of 30-60% Linear Sucrose Gradient

Five equally proportioned 1 ml increments from 60 to 30% sucrose (w/w) were layered one on top of the other. The tubes were then kept in the cold room overnight to allow diffusion.

0.25 ml of mitochondrial fraction was added to the top of the gradient; the tubes were then centrifuged at 39,000 rpm for 3 hours in the Beckman SW50L rotor. The contents of the tube were fractionated by pumping an 80% sucrose solution into the bottom of the tube and collecting equal fractions from the top of the tube which had been fitted with an adapter for this purpose. All enzyme and protein assays were run on these fractions.

2. Preparation of 20-50% Non-Linear Ficoll Gradient

A 50 ml non-linear ficoll gradient was set up as follows: 5 ml of 75% sucrose (w/w) was pipetted into the bottom of the tube. Ten ml of 50, 42.5 and 35% ficoll were layered one on top of the other in the order given. Nine and 6 ml of 27.5 and 20% ficoll respectively were added. The tubes were set in the cold room overnight.

Density gradient centrifugation of 20% ficoll-washed mitochondrial fraction was carried out for 25 minutes in the Sorvall SW-2 rotor at 10,000 rpm. The bands were collected by the same procedure as above.

D. Density Gradient Centrifugation of Thyroid Complete Mitochondrial Fraction

1. Preparation of 10-40% Linear Ficoll Gradient

Five ml of 65% sucrose were added to the bottom of the centrifuge tube to act as a cushion. Next, 9 ml each of 40, 32.5, 25, 17.5 and 10% ficoll were added in the order given, one on top of the other. The tubes were set in the cold room for a period not exceeding 18 hours. Sucrose and

ficoll solutions were prepared using 0.1 M potassium phosphate buffer, pH 8.0, with 1.0 mM EDTA and 0.1 mM KI.

One ml of the mitochondrial fraction was applied to the gradient which was then centrifuged for 20 minutes at 10,000 rpm in the Sorvall SW-2 rotor. Fractions were collected by use of the ISCO density gradient emptying apparatus.

2. Preparation of 10-40% Non-Linear Ficoll Gradient

Five ml of 65% sucrose were added to the bottom of the tube. Eleven, 10, 9, 8, and 7 ml of 40, 32.5, 25, 17.5 and 10% ficoll (w/w) respectively were added to the tube which was then set in the cold room for no longer than 18 hours. All solutions were made with 0.1 M potassium phosphate buffer-1.0 mM EDTA-0.1 mM KI. The centrifugation and fractionation method was the same as that employed in the linear density gradient.

E. Protein Determination

The method used was a variation of the Miller (1959) modification of the Lowry method (Lowry, et al. 1951).

1. Folin Phenol Reagent

The method of Folin Ciocalteau (1927) was used to prepare this reagent. One hundred g of Na_2WO_4-$2H_2O$ and 25 g of $Na_2MoO_4 \cdot 2H_2O$ were dissolved in 700 ml of deionized water. To this was added 50 ml of 85% phosphoric acid and 100 ml of concentrated HCl. This solution was refluxed for 10 hours after which time 150 g of Li_2SO_4, 50 ml of water and a few drops of liquid bromine solution were added. The solution was boiled for 15 minutes to remove excess bromine, cooled to room temperature, diluted to 1.0 liter and filtered through a sintered glass funnel. It was diluted 1:11 for use according to the Miller modification.

2. Alkaline Copper Reagent

This reagent was a modification of that described by Lowry (1951). The reagent was prepared by rapidly mixing 25 ml containing 0.2 g of $CuSO_4 \cdot 5H_2O$ with 50 ml containing 0.6 g of Tris-(hydroxymethyl)-aminomethane and diluting to 100 ml with distilled water. The resulting Tris-copper stock reagent was diluted 1:11 with 10% Na_2CO_3 in 0.5 N NaOH for use.

3. Protein Standard and Assay

A standard curve was constructed by making a 2 mg bovine serum albumin per ml solution and performing a protein assay on various concentrations of this solution.

The routine assay was run by mixing 1.0 ml of alkaline copper solution to 1.0 ml of protein solution (0.02-0.2 mg/ml) and allowing to stand for 10 minutes. At the end of this period 3.0 ml of the diluted Folin reagent was added, allowed to stand at room temperature for 30 minutes and read at 650 mμ in a Coleman, Jr. Spectrophotometer. The absorbance was referred to a standard curve (Fig. 3). This assay for protein was used for all determinations on liver fractions and as elsewhere noted in the text.

4. Ultraviolet Absorption at 280 mμ

Protein determinations of thyroid fractions which had been removed from density gradients were made by taking a reading at 280 mμ on a Coleman Autoset Spectrophotometer. Protein concentration was calculated using the extinction coefficient, $E^{1\%}_{cm} = 6.60$ at 280 mμ (Cohn, et al. 1947) which gave a protein concentration (mg/ml) equal to 1.515 times the absorbance at 280 m .

F. Lysosomal Enzyme Markers

1. Acid Phosphatase Assay

a. Standard Procedure

Acid phosphatase activity was determined according to the

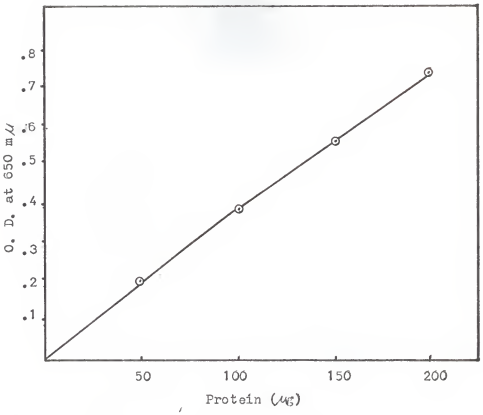

Fig. 3. Standard curve for protein analysis. Assay procedure as given under Methods, page 23.

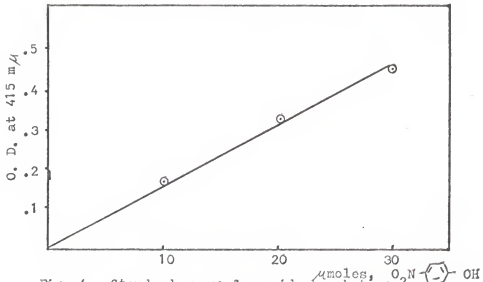

Fig. 4. Standard curve for acid phosphatase assay. Analysis procedure described under Methods, page 25.

method of Neil and Horner (1964). One ml of an appropriate dilution of the sample was incubated at 37° C for 10 minutes with 0.5 ml of 25 mM p-nitrophenylphosphate and 3.5 ml of 0.1 M sodium acetate buffer, pH 5.0. The tubes were pre-incubated at 37° C for about three minutes before the reaction was started by the addition of substrate. The reaction was stopped by the rapid addition of 10 ml of 0.04 N NaOH and the optical density of the liberated p-nitrophenol read on a Coleman Jr. at 415 mμ. The final concentration of the substrate in the reaction mixture was 2.5 mM. A unit was defined as that activity which produced one micromole of p-nitrophenol per minute at 37° C.

b. Standard Curve

A standard solution of 0.50 mM p-nitrophenol was prepared by dissolving 0.0696 g of p-nitrophenol, disodium salt, in 1000 ml deionized water. Various aliquots of this solution (0.2 to 1.0 ml) were added to 0.1 M sodium acetate buffer to a total of 5 ml of solution. Ten ml of 0.04 N NaOH were added to the tubes and read at 415 mμ. Assay readings were referred to the constructed standard curve (Fig. 4).

2. Acid DNAase Assay

This assay was based on the methods of Kunitz (1950) and measured the increased UV absorption at 260 mμ during the course of depolymerization of calf thymus DNA (0.4 mg). The method was adapted to include a lowering of the pH to 4.6 and a magnesium ion concentration to 1.0 mM. One-tenth molar sodium acetate buffered enzyme solution and substrate were pre-incubated separately at 37° C before being mixed together. Readings were taken on a Beckman DU every 15 seconds during the course of the reaction. One unit was defined as that activity which caused an increase in absorbancy of 0.001 per minute per ml enzyme at 37° C.

3. Protease Assay

a. Standard Procedure

One-half ml of enzyme solution was incubated at 37° C for 30 minutes with 2.5 ml of 1% hemoglobin, pH 3.5, prepared according to the method of McQuillan and Trikojus (1953). The reaction was stopped with 5 ml of 0.6 N trichloroacetic acid and centrifuged at 1,000 x g for 10 minutes. Five ml of supernatant were added to 2 ml of 3.0 N NaOH. To this was added 3 ml of 1:3 diluted Folin-Ciocalteau reagent. The absorbancy at 650 mμ was read immediately. This is a modification of the method of Anson (1938). One unit was defined as that activity which liberates 1 meq x 10^{-4} of tyrosine per 30 minutes.

b. Standard Curve

A standard tyrosine solution containing 6.56 x 10^{-4} milliequivalents per ml was made by dissolving 0.460 mg of tyrosine in 50 ml of 0.2 N HCl. Various aliquots of this solution (1 to 5 ml) were made up to a volume of 5 ml with deionized water. Two ml of 3 N NaOH were added, the tubes shaken and 3 ml of 1:3 diluted Folin reagent added. The absorbancy was read at 650 mμ within 2 minutes after the addition of the Folin reagent. Assay readings were referred to this standard curve for calculation of cathepsin activity (Fig. 5).

4. Amylase Assay

Four and nine-tenths ml of 0.10% soluble starch in 0.05 M potassium phosphate buffer were placed in a test tube. Two-hundreths ml of 5.1 x 10^{-3} N I_2 (1.02 x 10^{-4} equivalents) were added to the tube to give an optical density of 0.40 at 600 mμ. One-tenth ml of enzyme was added rapidly to the tube at time zero and the amount of time required for the optical density to fall to 0.1 was measured. This reaction was carried out

at 25° C. One unit was defined at that amount of enzyme which produced an optical density of 0.1 at 600 mμ in one second.

G. Mitochondria Enzyme Markers

1. Succinic Dehydrogenase Assay

a. Standard Procedure

The method used was essentially that of Nachlas, et al. (1960). One-half ml sodium succinate (0.2 M), 1.95 ml of 0.1 M potassium phosphate buffer, pH 7.7, 1.0 ml of 0.2% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) and 0.5 ml of 0.1% gelatin were preincubated at 37° C for five minutes. After this time 50 μliter of enzyme and 0.5 ml of phenazine methosulfate (0.8%) were added and incubation continued for 15 minutes. The reaction was quenched by the addition of 0.5 ml of 0.25 M HCl. The optical density of the resultant solution was read at 540 mμ on a Coleman, Jr. Spectrophotometer. Boiled blanks were carried along with the assay. One unit was defined as that activity which caused the formation of one μg of formazan per minute. In this colorimetric reaction, phenazine methosulfate acted as an artificial electron carrier between succinic dehydrogenase and oxygen. Gelatin was used to disperse the INT, which upon reduction becomes insoluble. In view of the known instability of succinic dehydrogenase in purified particles, this assay was always run immediately after fractionation of the tube contents.

b. Standard Curve

Succinic dehydrogenase, which is commercially unavailable, was purified according to an abbreviated procedure suggested by Singer, et al. (1962) by rat liver mitochondrial isolation, differential extraction, solubilization with pH 10.3 glycine buffer, neutralization with Dowex 50-H cycle, and calcium phosphate gel adsorption. The eluate received from the calcium

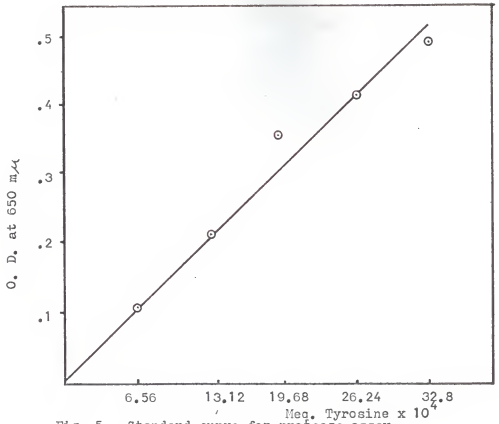

Fig. 5. Standard curve for protease assay. Analysis procedure described under Methods, page 21.

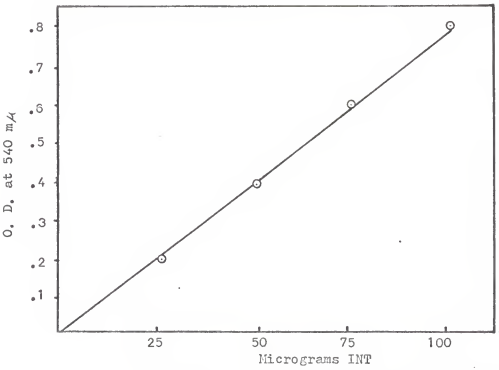

Fig. 6. Standard curve for succinic dehydrogenase assay. Analysis described under Methods, page 29.

phosphate gel was used for preparing a standard curve. A solution of INT was prepared in 0.1 M phosphate buffer, pH 7.7, such that 1.0 ml contained 0.25 mg INT. Varying amounts of the formazan solution (0.2 to 1.0 ml) were added to the tubes and made up to a volume of 2.5 ml with buffer. To these tubes was added 0.5 ml of 0.1% gelatin. The tubes were then allowed to pre-incubate at 37° C for 5 minutes after which time 1.0 ml of buffer containing 2 mg of NADH, 0.5 ml of 0.8% PMS and 0.5 ml enzyme solution were added. After 10 minutes of incubation the reaction was quenched with 0.5 ml of 0.25 N HCl and the tubes read in a Coleman, Jr. at 540 mμ. A standard curve was constructed from the data (Fig. 6) and all assays were referred to this curve for a calculation of activity.

c. Addition of Activators

In view of the known inhibitory effect of oxaloacetic acid on succinic dehydrogenase activity, cysteine sulfinic acid (9 mM) was added to the assay media as suggested by Singer and Lusty (1961). This amino acid is considered to be the most effective agent for the removal of oxaloacetic acid for the rapid and stoichiometric removal of oxaloacetate from mitochondrial systems (Singer and Kearney, 1955). Three-quarters mM calcium was added to break down the permeability barrier of the mitochondria membrane to the dye INT (Cleland and Slater, 1953). The addition of these activators was used in half of the assays only and is noted in the text.

2. Cytochrome Oxidase Assay

Measurement of cytochrome oxidase activity was carried out as a modification of the method of Cooperstein and Lazarow (1951). Cytochrome c (type III) was made up to a concentration of 5 mg/ml with 0.1 M potassium phosphate buffer, pH 7.4. The substrate was reduced by adding a few crystals of $Na_2S_2O_3$ to the solution. Excess reducing agent was removed by bubbling in

oxygen. The assay itself was carried out by adding 25 μl of enzyme solution from the gradient to a cuvette containing 2.8 ml of pH 7.4 phosphate buffer and 0.2 ml of the reduced substrate solution and measuring the initial decrease in optical density at 550 mμ as a function of time. The readings were taken on a Coleman Autoset Spectrophotometer. Activity was expressed as delta O. D./ minute at 550 mμ.

H. Peroxisome Enzyme Markers

1. Catalase Assay

Catalase activity was determined spectrophotometrically at 240 mμ by a modification of the method of Beers and Sizer (1952) in which the disappearance of hydrogen peroxide was followed at pH 7.0. To 2.95 ml of diluted enzyme-phosphate buffered solution was added 0.05 ml of 0.9 M H_2O_2 at 25° C. The initial rate of H_2O_2 disappearance was followed on a Beckman DU. One unit was defined as one micromole of H_2O_2 decomposition per minute at 25° C. Activity was expressed in the text as delta O.D./minute at 240 mμ. The final concentration of hydrogen peroxide was 15 mM.

2. Uricase Assay

Uricase activity was determined by adding 1.0 ml of enzyme solution to 2.0 ml of substrate solution containing 20 μg of uric acid in 0.1 M borate buffer, pH 8.5 and measuring the decrease in absorbancy at 290 mμ. The assay was carried out at 25° C and followed in a Beckman DU. One unit was defined as that activity which converts uric acid to allantoin at the rate of 1 μmole/minute at 25° C.

3. Peroxidase Assay

a. Standard Proccdure

The peroxidase assay employed was that of the Nicholson (1967) modification of the method of Chance and Maehly (1954) utilizing

guaiacol as substrate. The reaction was carried out in the following manner: 4.9 ml of 0.1 M Tris-HCl buffer, pH 7.8, 0.1 ml enzyme solution and 1.0 ml of 0.1 M guaiacol were mixed in a test tube and warmed to 30° C in a water bath. The tubes were placed in a Coleman, Jr., the absorbancy adjusted to zero at 470 mμ and then 1.0 ml of 1.4 mM H_2O_2 quickly blown into the tube and shaken. The time for the optical density to come to a value of 0.1 at 470 mμ was recorded. An enzyme unit was here defined as that amount of enzyme which produced an absorbance of 0.1 at 470 mμ in one second. Final concentrations of guaiacol and H_2O_2 were 14.3 mM and 0.2 mM respectively.

b. Hydrogen Peroxide Concentration

H_2O_2 reacts quantitatively with H_4TiO_4 (TiO_2 in H_2SO_4) to produce a yellow color which is proportional to H_2O_2 concentration and is stable for 6 hours (Eisenberg, 1943). A solution of 0.0784 g $K_2Cr_2O_7$ per liter of water has a color equal to that produced by reacting 1 ml of 1.4 mM H_2O_2 with 5 ml of titanium sulfate reagent. Standard dichromate solution was placed in a colorimeter tube and the absorbance determined at 410 mμ using a water blank. A 0.3% H_2O_2 stock solution was diluted with deionized water until the reaction of one ml of the solution with 5 ml of titanium reagent produced an absorbance equal to that of the standard dichromate solution at 410 mμ. (Fig 7) The titanium sulfate reagent was prepared by digesting 1 g of reagent grade TiO_2 with 100 ml of concentrated H_2SO_4 for 16 hours at 150° C, cooling and diluting to 500 ml and filtering through a sintered glass funnel. This stock solution was diluted 1:8 with distilled water for use.

I. Complete Peroxidase Activity Survey

Frozen hog thyroid glands were thawed and put through the usual fractionation scheme for the collection of mitochondrial fraction. Guaiacol

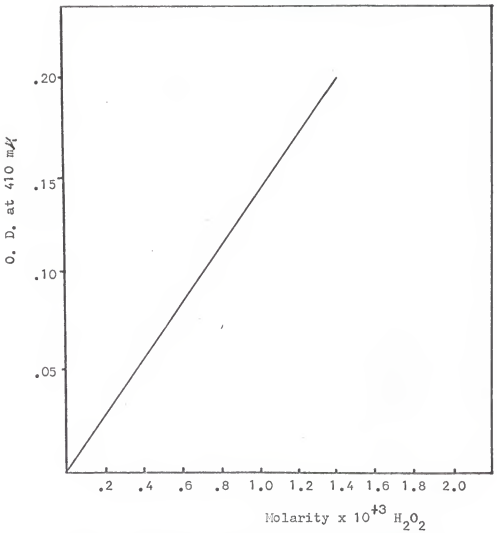

Fig. 7. Standard curve for H_2O_2 determination. Analysis procedure described under Methods, page 31.

assays and protein determinations were performed on the crude homogenate, 1,000 x g homogenate, mixed nuclear-debris fraction, 39,000 x g supernatant and mitochondrial pellet. In addition, the nuclei were separated from the cell debris by layering the mixed fraction on top of a 0.1 M phosphate buffered 43.8% sucrose-0.1 mM KI solution and centrifuged at 39,000 x g for 30 minutes. The nuclei sedimented to the bottom of the tube while the debris remained on top. The ribosomal fraction was obtained by centrifuging the 39,000 x g supernatant at 100,000 x g for 60 minutes in the Beckman L2-65 ultracentrifuge. A diagram showing the complete fractionation scheme and points of assay is presented in Figure 8.

J. Preservation of Complete Mitochondrial Fraction Peroxidase Activity

Frozen hog thyroids were subjected to the usual mitochondrial fraction collection method. (Methods, page 18). The ficoll wash step was deleted. The complete mitochondrial fraction was then resuspended with 4.17 volumes of 0.1 M potassium phosphate buffer, pH 8.0--0.1 mM KI--1.0 mM EDTA. A guaiacol assay for peroxidase activity and a protein determination were taken immediately at this point. Protein concentration was assumed to be constant throughout the experiment. The remaining portion of the resuspended CMF was divided into four aliquots and treated according to the following scheme:

where a-- CMF plus buffer solution (1:1)
b-- CMF plus 2% thyroglobulin in buffer (1:1)
c-- CMF plus 2% gelatin in buffer (1:1)
d-- CMF plus buffer (1:1) under N_2 atmosphere.

Guaiacol assays for peroxidase activity were run at various time intervals up to 45 hours after resuspension in order to determine the degree of

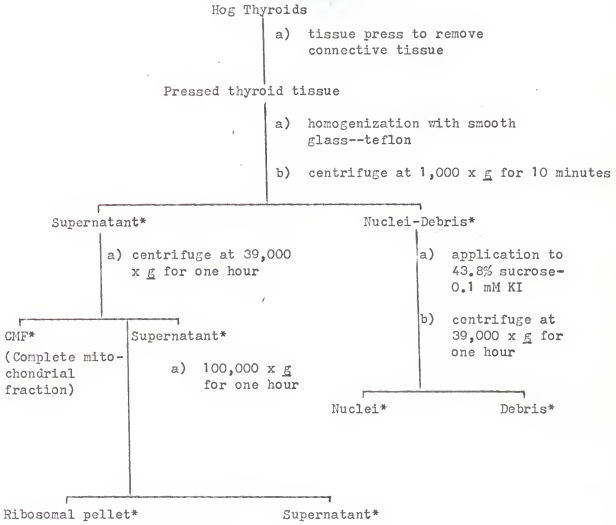

Fig. 8. Complete fractionation scheme for thyroid tissue. (* marks points of protein determinations and peroxidase assay).

inactivation of the enzyme.

This work was prompted by the previous observation that as much as 95% of the thyroid peroxidase activity was lost from this fraction in a 24 hour period if the particles were resuspended in either 0.25 M sucrose-1.0 mM EDTA or in 0.1 M sodium phosphate buffer, pH 8.0-0.1 mM KI before freezing (Nicholson, 1967). If the particles were not resuspended, no loss of activity occurred for months. It had been assumed by the author of this text that if resuspension was not carried out on the mitochondrial fraction, that it could be run through a density gradient, the fractions collected and stored immediately without loss of peroxidase activity. This was found not to be the case.

K. Determination of Multiple Thyroid Peroxidase

1. Collection of Peroxidase Active Particles

Fresh hog thyroids were put through the complete fractionation scheme for the collection of the mitochondrial fraction including the ficoll wash (Methods, page 18). The washed particles were centrifuged on a 50 ml, 10 to 40% non-linear ficoll gradient as described under Methods, page 22. Fractions were collected from the gradient and each tube was immediately analyzed for peroxidase activity, cytochrome oxidase activity and protein content. After the bands were identified as to enzyme and protein content, a second density gradient experiment was run identical to that described above. The tubes containing the predetermined mitochondrial and peroxisomal peroxidase activity were pooled such that there were 8 ml of each of the two bands.

2. Treatment of Peroxidase Containing Fractions

The two major fractions were each subdivided into four fractions and treated according to the following scheme:

where a is the control (2 ml band material plus 2 ml phosphate buffer-1.0 mM EDTA-0.1 mM KI); b is the same as "a" with N_2 atmosphere added; c consists of 2 ml of band material plus 2 ml of 2% thyroglobulin; and d consists of 2 ml of band material plus 2 ml of 2% gelatin.

Immediately after the bands were taken from the gradient and pooled, a protein determination and peroxidase assay were made. Protein content was assumed to be constant and the peroxidase assay gave the activity of the enzyme at time zero off the gradient. Hereafter, peroxidase assays were made on each sample at various times up to 60 hours after removal from the gradient.

L. Electron Microscopic Analysis of Liver Subcellular Particles

1. Preparation of Sections from Density Gradient Fractions

The light and heavy mitochondrial-lysosomal bands and the peroxisomal band were collected from a 20-50% ficoll density gradient. These three bands were recentrifuged to a pellet, drained of ficoll and mixed with as small amount as possible of 1% agar. The agar dispersed pellets were allowed to harden in the refrigerator, cut into thin slices and fixed for 1 hour in 0.066 M s-collidine buffered 4% paraformaldehyde at pH 7.4 (Lynn, et al., 1966). The agar pellets were next post fixed in s-collidine buffered 1% osmium tetroxide for one day. Dehydration was achieved by 10 minute exposures to 70% and 95% ethanol followed by two 15 minute exposures to 100% ethanol. Propylene oxide was used as the transitional solvent--15 minutes. Infiltration was achieved with propylene oxide-DER 732-332 in a ratio of 1:1. Embedding was achieved in size "00" gelatin capsules in which polymerization of the epoxy resin was accomplished with a graded series of temperature of 37° C, 45° C and 60° C of an approximate duration of 18 hours each (Lockwood

and Langston, 1964).

The hardened blocks were cooled to room temperature, trimmed into pyramids and thin sectioned with a 50 degree angle glass knife. An LKB Ultratome was used for this purpose. Thin sections of approximately 600-900 Angstroms were produced as determined by interference colors. The sections were recovered on 200 mesh Athene-type parlodion coated grids. After drying, the mounted sections were stained as described in the various legends. Electron micrographs were taken on the RCA EMU-2D.

2. Histochemical Analysis of Lysosomal Fractions

The Gomori acid phosphatase reaction mixture was made by dissolving 0.6 g of lead nitrate in 500 ml of 0.05 M sodium acetate buffer, pH 5.0, and 50 ml of 3% sodium glycerophosphate. The mixture was warmed in a 37° C water bath for about 4 hours and filtered. The lysosome-containing bands were subjected to this reaction mixture for 10 minutes after paraformaldehyde fixation (Gomori, 1952). The pellets were rinsed twice with distilled water, once in 1% acetic acid and finally in dilute $(NH_4)_2S$ to produce the electron dense PbS resulting from enzymatic activity. The acid rinse was used to remove protein bound lead. Enzymatically produced lead phosphate is not removed by the acid rinse (Gomori, 1952). All further steps were carried out as in the preceding section.

M. Electron Microscopic Analysis of Thyroid Subcellular Particles

1. Preparation of Sections from Density Gradient Fractions

The bands from a 10-40% non-linear ficoll gradient were fixed in 0.066 M s-collidine buffered 4% paraformaldehyde for 1 hour. These fractions were then centrifuged to a pellet and set in 1% agar to harden. The agar was next cut into slices and post-fixed in 1% OsO_4 buffered with s-col-

lidine, dehydrated in graded alcohol solutions as before and carried through the remaining procedure identical to that employed for the preparation of liver fractions (Methods, page 36). It will be noted that the fixation preceeded the agar embedment procedure with work on thyroid fractions. This was done to better preserve the structural components of the particles.

2. Preparation of Thyroid Slices

Hog thyroids were excised and sliced into 1 mm cubes within a couple of minutes after death. The slicing was done in pH 7.4 0.066 M s-collidine buffered 4% paraformaldehyde. A portion of these slices were saved for histochemical work. The cubes were rinsed free of fixative and subjected to 1% OsO_4 postfixation for 1 hour. The cubes were then put through the usual dehydration, infiltration and embedment procedures. Embedment was achieved in DER 732-332 mixture. The thin slices were caught up by Athene type 400 mesh parlodion coated grids.

3. Benzidine Staining of Thyroid Slices

The method used to stain for peroxidase activity was essentially that of Schneeberger-Keeley and Karnovsky (1968). The benzidine stain was prepared by dissolving 5 mg of 3,3'-diaminobenzidine in 10 ml of 0.1 M Tris-HCl buffer, pH 7.6. A portion of the fixed thyroid slices from the preceeding section were subjected to this stain for 10 minutes at room temperature, after which time 0.03 ml of 10% H_2O_2 was added to the mixture and allowed to react for 20 minutes at room temperature with frequent shaking. The slices were then rinsed with deionized water and carried through the remaining procedure for preparation of slices (Methods, page 37). The final concentration of H_2O_2 was approximately 1 mM.

RESULTS AND DISCUSSION

I. Enzyme Distribution of Subcellular Particles in Liver

A. Distribution from Linear Sucrose Gradient

Figure 9 shows the enzyme distribution obtained by density gradient centrifugation of rabbit liver subcellular particles on a 30-60% linear sucrose gradient. These values are the average of six determinations. Of special interest in this investigation was the apparent dual distribution of catalase. The mitochondrial fraction did appear to contain a catalase component, whose activity was too large to be accounted for by assuming a leakage from peroxisones or to membrane rupture. Catalase activity was in all 6 experiments found in the heavy mitochondrial peak as determined by protein content and succinic dehydrogenase activity. Had leakage or membrane rupture occurred, the released soluble catalase would have appeared in the first fraction of the gradient and not consistently at an intermediate value. Although many authors have previously supported the idea that catalase was a mitochondrial enzyme, this was the first time that it has been shown to sediment on a density gradient with these particles. This may reflect a species difference, however, since previous work has been done with rat or mouse liver and not with rabbit liver.

Some degree of difficulty was had in using succinic dehydrogenase as a mitochondrial marker in these assays due to its apparent low activity and rapid inactivation once the particles had been purified. The finding of Singer and Lusty (1960) that the addition of calcium and cysteine sulfinic acid (CSA) enhanced the activity of the assay by the breakdown of the permeability of the membrane to the dye, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT), and by the removal of oxaloacetic acid inhibition was therefore utilized in three different assays. The results were compared

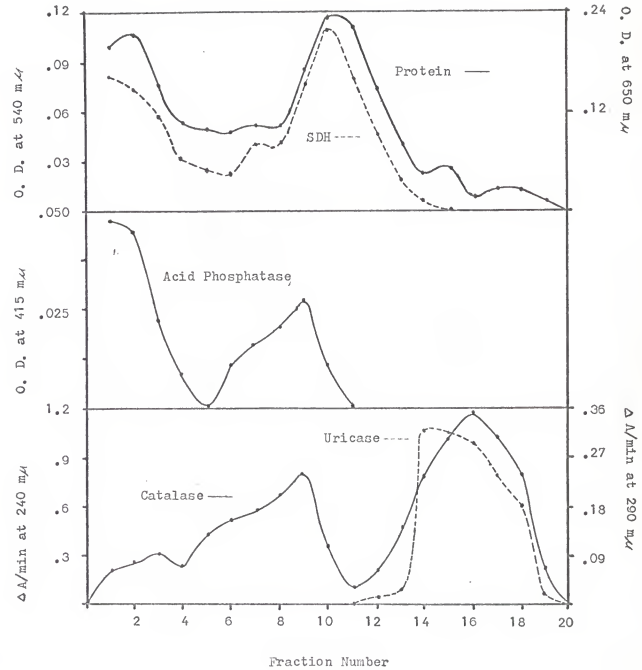

Fig. 9. Enzyme distribution of liver subcellular particles on a linear sucrose gradient.

with those from previous experiments which did not contain the two activating constituents and are depicted graphically in Figure 10. Activity values are given in Table I. As can be seen from Figure 10, the enzyme and protein distributions from the two experiments are essentially the same. Examination reveals that whereas more protein sedimented into the heavy mitochondrial fraction in the second group of experiments than in the first group, this protein also exhibited more succinic dehydrogenase absolute activity. The inclusion of calcium and CSA into the assay incubation media was therefore active in increasing the specific activity of the assay as indicated in Table I.

B. Distribution from Non-Linear Ficoll Gradient

It was found from previous experiments with ficoll density gradients that a linear gradient did not give a clear separation of the light and heavy mitochondria and lysosomes and that only two bands were obtained, one containing all populations of mitochondria and lysosomes and one the peroxisomes. For this reason, a non-linear gradient was tested for its applicability to the separation procedure. The results of utilizing a non-linear ficoll gradient are depicted graphically in Figure 11. In contradiction to earlier reports (Beaufay, et al. 1964), the substance ficoll offers as good, if not better resolution of liver complete mitochondrial fractions as sucrose, which has up to this time been classically employed.

1. Effect of Ficoll on Equilibrium Density of Subcellular Particles

Comparison of Figures 9 and 11 indicates that the substance ficoll had the following effects on the enzyme distribution of the complete mitochondrial fraction from rabbit liver:

> a shifting of total protein and succinic dehydrogenase in the light mitochondrial fraction toward a region of higher equilibrium density;

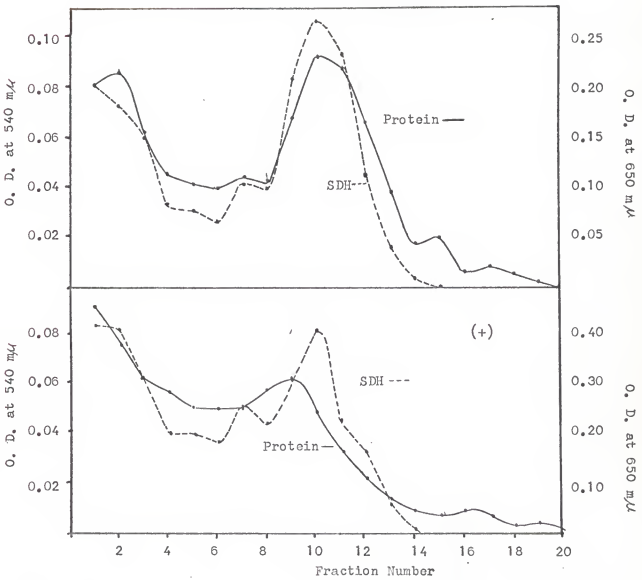

Fig. 10. Effect of addition of calcium and CSA on succinic dehydrogenase activity in liver subcellular particles.

(+) Indicates calcium and cysteine sulfinic acid added.

TABLE I

EFFECT OF CALCIUM AND CSA ON SDH ACTIVITY IN PEAK FRACTIONS FROM DENSITY GRADIENT

Fraction Number	Protein mg/ml	Units/ml	Spec. Act. units/mg	% Act.
1 (+)	2.00	20.0	10.00	186.5
1 (-)	4.48	24.0	5.36	100.0
10 (+)	2.36	28.0	11.86	184.7
10 (-)	3.12	20.0	6.42	100.0

(+) indicates that Ca^{++} and CSA have been added to media
(-) indicates assay conducted in the absence of Ca^{++} and CSA

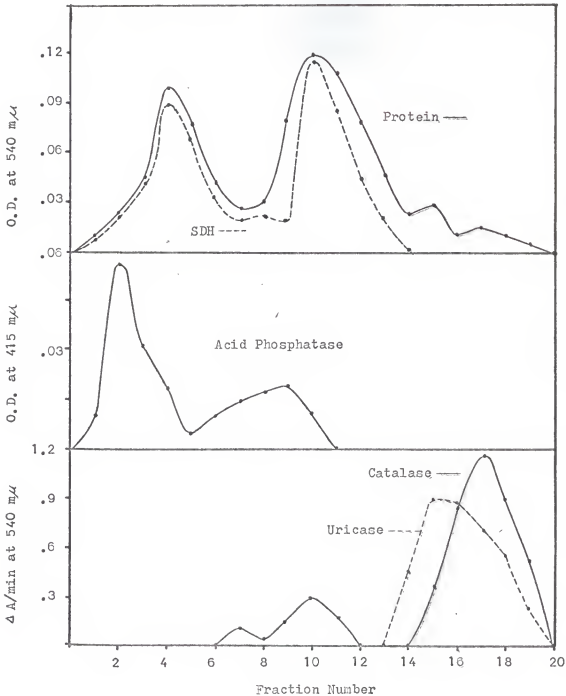

Fig. 11. Enzyme distribution of liver subcellular particles on a non-linear ficoll gradient.

the restricting of larger amounts of lysosomal enzyme in the less dense regions of the light mitochondrial fraction;

a shifting of both uricase and catalase activity in the peroxisomal region toward a region of higher density; and

a shifting of low density catalase activity directly into the heavy mitochondrial fraction.

The experiment therefore confirms the finding of Beaufay, et al. (1964) that the substance ficoll appears to cause a slight increase in total protein and mitochondrial enzyme protein as exhibited in the shifting to the right of both succinic dehydrogenase and catalase activity.

Both of the peroxisomal enzymes, catalase and uricase, are also shifted toward a region of higher equilibrium density, thus attaining an equilibrium position even further away from the lysosomal enzymes. Although it has not previously been reported that ficoll has any effect on the equilibrium density of the lysosomal particles, this investigation tends to point to the fact that more lysosomal particles are restricted to regions of lower equilibrium density in this substance than in the classical sucrose gradient.

2. Effect of Ficoll on Uricase Activity

Table II compares the uricase activity exhibited on a sucrose gradient and on a ficoll density gradient. These values were taken from the fraction of maximum uricase activity. Even though the protein content is approximately the same in both fractions, there is a 7.3 fold decrease in absolute activity and a 9.4 decrease in specific activity of uricase in the ficoll gradient. This cannot be accounted for by a leaching out of uricase protein from the peroxisomes into the supernatant since uricase activity is associated with the insoluble core. It must be assumed therefore that the substance ficoll has in some way damaged the crystalline core to destroy uricase activity. This may be nothing more than a conformational change

TABLE II

URICASE ACTIVITY IN PEAK FRACTIONS
FROM SUCROSE AND FICOLL GRADIENTS

	Protein content mgm/ml	Activity unit/ml	Specific Activity unit/mgm
From sucrose gradient	0.44	8.11	18.50
From ficoll gradient	0.56	1.11	1.98

TABLE III

MITOCHONDRIAL CATALASE ACTIVITY EXHIBITED
ON FICOLL AND SUCROSE GRADIENTS

	Protein content mgm/ml	Activity unit/ml	Specific Activity unit/mgm
From sucrose gradient	1.72	5.24×10^3	3.04×10^3
From ficoll gradient	2.48	2.14×10^3	8.64×10^2

about the active site or a disruption of the crystalline structure of the core.

3. Effect of Ficoll on Mitochondrial Catalase Activity

Catalase as contained within the mitochondrial band also suffered a deactivation or loss in specific activity in the ficoll gradient. This is shown in Table III. Although the protein content is essentially the same on the ficoll gradient as on the sucrose gradient, the mitochondrial catalase has suffered a 3.5 fold decrease in specific activity. Peroxisomal catalase activity was essentially the same in both instances. These facts indicate that mitochondrial catalase is actually different from peroxisomal catalase and that this is a true difference in structure and enzymatic properties between the enzymes as contained within the two different subcellular particles.

II. Enzyme Distribution of Subcellular Particles in Thyroid

A. Distribution from Fresh Thyroids on Linear Ficoll Gradients

The enzyme and protein distribution obtained from fresh thyroid mitochondrial fraction on a 10-40% linear ficoll gradient is depicted in Fig. 12.

B. Distribution from Fresh Thyroids on Non-Linear Ficoll Gradients

Fig. 13 depicts the subcellular enzyme and protein distribution of fresh hog thyroids on a non-linear ficoll gradient. As can be seen, amylase followed the same distribution as acid phosphatase and it is postulated at this point that this enzyme may well be contained within the lysosomal particles.

1. Disadvantages of Linear Density Gradient

A comparison of the subcellular enzyme distribution from fresh thyroid tissue on the linear ficoll gradient (Fig. 12) with that from

a non-linear ficoll gradient (Fig. 13) shows that the use of a linear gradient had certain disadvantages in obtaining a good separation of subcellular components. There was no differentiation of lysosomal particles achieved; i.e., all populations and species of lysosomes were concentrated in one band as exhibited by acid phosphatase distribution. Widespread contamination of protein and lysosomal and mitochondrial enzyme activities had occurred in the dense region of the gradient. Cytochrome oxidase, amylase, acid phosphatase activities were recorded in the uricase and catalase activity peaks along with a considerable amount of protein. Mitochondrial protein was contained within one rather wide band with little or no differentiation of light and heavy mitochondria as exhibited by cytochrome oxidase activity. Basically, a linear ficoll gradient seemed to be inadequate for the separation of different species of the same subcellular component.

2. Effect of Non-Linear Gradient

Figures 12 and 13 show that the subcellular particles isolated from fresh thyroid exhibited a more definitive and refined distribution on a non-linear gradient than on a linear gradient. The major protein band was considerably narrower and light and heavy mitochondria, as exhibited by cytochrome oxidase activity, were cleanly separated. Lysosomal particles were differentiated into all three species which are known to exist--light, medium and heavy density. This differentiation allowed for the hypothesis that amylase was probably contained within the lysosomal membrane since its activity exactly paralleled that of acid phosphatase activity which is known to be contained within the lysosome. There was a shifting to the right of catalase, uricase and peroxidase activities, with the result that a wider separation of peroxisomes from lysosomes and mitochondria was achieved.

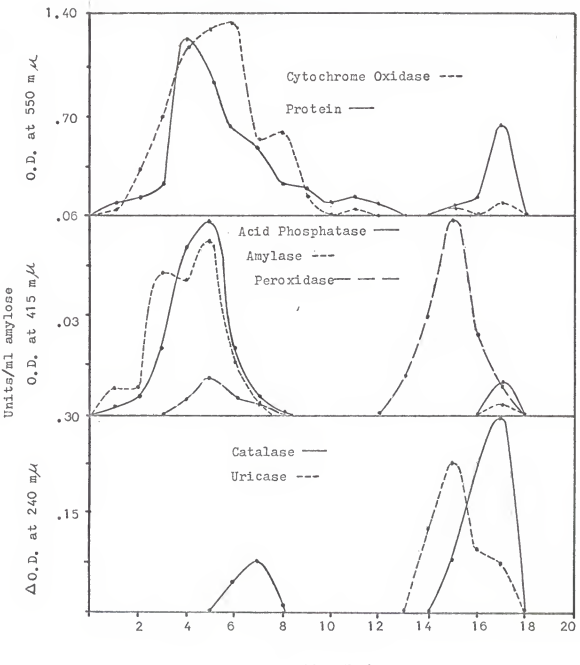

Fig. 12. Enzyme and protein distribution from fresh thyroids on linear ficoll gradient.

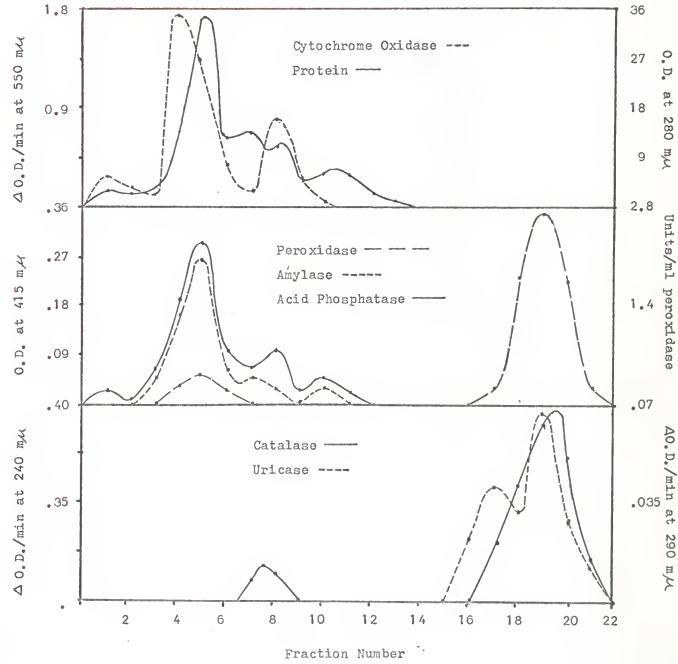

Fig. 13. Enzyme and protein distribution from fresh thyroids on non-linear ficoll gradient.

C. Distribution from Frozen Thyroids on Non-Linear Ficoll Gradient

Figure 14 depicts the protein and enzyme distribution obtained after density gradient centrifugation of frozen thyroid mitochondrial fraction on a non-linear ficoll gradient.

1. Advantages of Employing Fresh Tissue

Comparison of the enzyme and protein distributions from frozen thyroid tissue on a non-linear gradient with that obtained from fresh thyroid tissue revealed that the use of fresh tissue offered certain immediate advantages. There is a sharper separation of light and heavy mitochondrial cytochrome oxidase activity. Lysosomes are resolved into all three density species instead of two exhibited with frozen tissue. The light lysosomal specific activity was maintained to a greater extent. This may possible be accounted for by the fact that freezing and thawing is known to rupture the lysosomal membrane thus releasing its contents into the soluble fraction (De Reuck, 1963).

The distribution obtained from frozen tissue on a non-linear ficoll gradient was similar to that obtained with fresh tissue on a linear gradient. Both showed poor resolution of light and heavy mitochondrial activity and an inability to separate lysosomal particles. This is most often noted in experiments performed on a classical sucrose gradient. Ficoll seemed to be particularly well suited for this purpose. Resolution of subcellular particles can be achieved with a refinement that apparently cannot be had on sucrose gradients.

2. Disappearance of Heavy Lysosomal Activity

Since the dense lysosomes appear to be contained in a region of relatively low protein density to begin with (see Fig. 13) this may help to account for the complete disappearance of this band in the frozen particles.

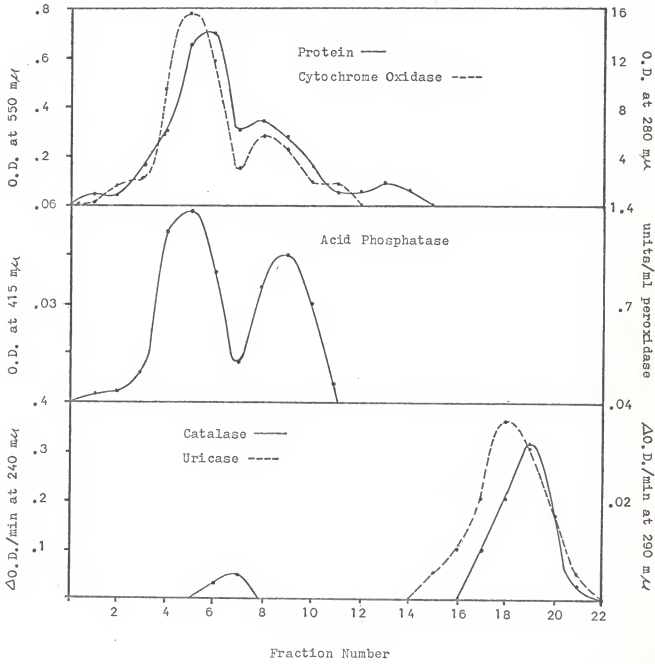

Fig. 14. Enzyme and protein distribution from frozen thyroid on non-linear ficoll gradient.

It must be assumed that the freezing and thawing process would cause the breakdown of a certain percentage of all species of lysosomes. Since the dense lysosomes appear to comprise a definite minority, the breakdown of the membrane with the release of lysosomal enzyme would be most apparent in this area. This activity would have then been discharged into the supernatant during the isolation procedure. A chart showing the correlation of lysosomal activity in the isolated bands is presented in Table IV for further clarification of this point.

D. Peroxisomal Peroxidase

A major conclusion to be drawn from the group of density gradient experiments is that thyroid peroxidase resides mainly in the peroxisomes. It was consistently found to sediment with a rather high specific activity in a region of high density together with uricase and catalase. It is assumed at this point to constitute a part of the "sap" of these particles, and is suggested to be the thyroid peroxidase-iodinase described by Nicholson (1967) which catalyzes the oxidation of iodide and then utilizes the oxidized iodide to catalyze the iodination of tyrosine groups. It is further suggested that the requisite formation of H_2O_2 in this particle occurs through the action of uricase and that catalase may possibly act as a protective mechanism in the prevention of an over accumulation of H_2O_2 which is not needed by the peroxidase-iodinase. This would most likely function when the demand for thyroxine is low.

E. Mitochondrial Catalase and Peroxidase

Catalase was consistently recorded in the lighter portion of the heavy mitochondrial peak and it is suggested that this enzyme is actually associated in some manner with the mitochondria and does not represent fragments resulting from mechanical disruption of the peroxisomes. This was

TABLE IV

ACID PHOSPHATASE ACTIVITY IN LYSOSOMAL PEAKS

Band	Protein mg/ml	Activity units/ml	Sp. Act. units/mg
Frozen:			
Light	21.01	3.70	0.177
Medium	9.55	2.70	0.283
Heavy	1.82	--	--
Fresh:			
Light	52.11	17.0	0.327
Medium	17.57	5.6	0.319
Heavy	8.48	2.8	0.331

TABLE V

SUBCELLULAR DISTRIBUTION OF THYROID PEROXIDASE

Step	Protein mg/ml	units	units/ml	units/mg	total units	% 1000 x g homog activity
Crude homog	33.2	.0470	0.1880	0.0057	4.24	197
1000 x g homog	29.6	.0299	0.0897	0.0030	2.15	100
Nuclear debris	7.0	.0168	0.0840	0.0129	1.75	81
Supernatant	24.0	.0254	0.0254	0.0011	0.533	25
Mito. Pellet	84.9	.0362	1.3030	0.1540	1.303	61
Nuclei	9.6	.0302	0.1510	0.0157	0.278	13
Debris	5.0	.0547	0.1090	0.0219	0.146	7
True supernatant	8.0	.0114	0.0114	0.0014	0.182	8
Ribosomal pellet	4.8	.0388	0.0388	0.0081	0.019	0.9

based partly on the fact that uricase was not found to contaminate this portion of the gradient. A portion of the thyroid peroxidase was also found in the peak of the light mitochondrial fraction and was assumed to be associated with it. It will be noted that this was also the peak of the light lysosomes. However, since peroxidase activity did not show up in either of the other two lysosomal peaks, the enzyme is with all likelihood not associated with these particles. It would be unfounded to assume such a highly selective mechanism which would allow for the inclusion of this enzyme only in this one species of lysosomes. A functional difference in the three species of lysosomes has never been postulated. Furthermore, lysosomal enzymes function in the acidic pH range and it is therefore difficult to imagine the inclusion of a pH 7.8 maximum activity enzyme in these particles.

III. Complete Peroxidase Activity Survey

Table V on page 54 gives a complete peroxidase activity analysis as the thyroid is put through a classical fractionation experiment (Methods, page 34). Consideration of the total number of units present in each fraction shows that activity was lost as the fractionation proceeds. All evidence now indicates that peroxidase activity is contained within the peroxisome and mitochondrion. It would be expected therefore that while the enzyme was associated with either of these particles in which it resides, it should be relatively stable. This is in fact what is found. The total units found in the 1,000 x g homogenate plus those in the nuclear-debris fraction should be equal to the total units in the crude homogenate. The actual value was 3.90 units which indicated a loss of 0.34 units. Likewise, the sum of the total units from the supernatant and the mitochondrial pellet (1.836 units) should add up to the value from the 1,000 x g homogenate (2.150 units). A loss of 0.314 units was noted at this step in the procedure. The largest loss in

units was observed for the sum of the nuclei and debris (0.424 units) as compared with a value of 1.750 units obtained for the combined fraction. It is suggested that this large loss in activity occurred due to the fact that peroxidase is represented in these fractions primarily as a contaminant. The values for the ribosomal pellet plus the true supernatant added up to only 0.201 units whereas the value for the supernatant came to 0.533 units. It appeared therefore that as peroxisomes and mitochondria were deleted from the system, peroxidase activity also disappeared.

IV. Maintenance of Total Mitochondrial Fraction Peroxidase Activity

A. The Effect of Added Constituents

The effect of the inclusion of 1% gelatin, 1% thyroglobulin and nitrogen atmosphere to the complete mitochondrial fraction is shown in Fig. 15 and Table VI. Figure 15 exemplifies the instability of peroxidase in that the majority of activity has been lost within the first five hours after resuspension in all samples except those which contain 1% thyroglobulin. There was little loss in activity in the other samples after this time. The samples containing thyroglobulin, however, appeared to have suffered a much smaller and slower loss of activity. Those samples containing 1% gelatin retained initial activity up to about four hours better than the control and that sample under N_2 atmosphere, but then underwent a rapid loss of activity within the next hour. It is suggested that the major portion of activity which Nicholson (1967) noticed as being lost at the end of the 24 hour period after resuspension had actually been lost within the first five hours and that as much as 73% of this activity can be retained by the inclusion of 1% buffered thyroglobulin. This was a considerable retention of activity as compared to the meager 5% retention previously recorded.

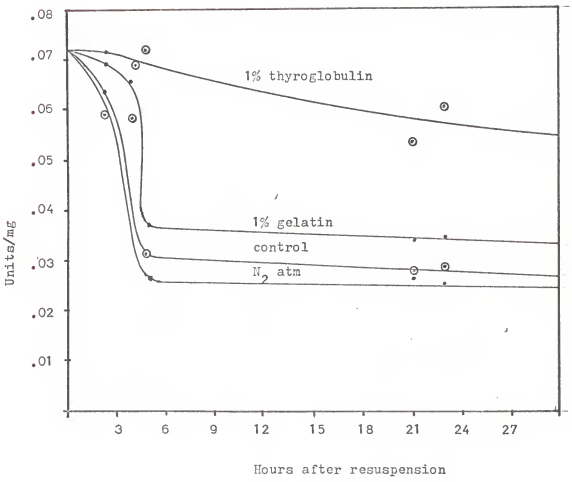

Fig. 15. Effect of treatment of CMF on peroxidase activity.

TABLE VI

SPECIFIC ACTIVITY OF PEROXIDASE IN TREATED CMF

	Control	N_2 atmosphere	1% Thyroglobulin	1% Gelatin
Sp. Act. t-0	0.0729	0.0729	0.0729	0.0729
% Loss t-0				
Sp. Act. t- 5 hr.	0.0315	0.0267	0.0700	0.0374
% Loss t-5 hr.	56.8	63.4	4.2	48.7
Sp. Act. t-24 hr.	0.0280	0.0240	0.0580	0.0340
% Loss t-24 hr.	61.6	67.1	20.6	53.4
Sp. Act. t-45 hr.	0.0220	0.0210	0.0531	0.0327
% Loss t-45 hr.	69.8	71.2	27.2	55.2

B. Effect of Dilution with Buffer

According to Nicholson (1967), 95% of the peroxidase activity was lost in the 24 hour period after resuspension of the CMF in 0.25 M sucrose or in 0.1 M potassium phosphate buffer, pH 8.0-0.1 mM KI. This was not borne out by this experiment. The highest loss in activity observed at 45 hours after resuspension was 69.8 for the control. However, the following modifications were employed:

Use of smooth glass Potter Elvejhem homogenizer as opposed to use of Waring blendor;

use of 0.77 M sucrose-0.1 mM KI-1.0 mM EDTA in 0.1 M potassium phosphate buffer for homogenization as opposed to 0.25 M sucrose-1.0 mM EDTA;

centrifugation at 39,000 x g for 1 hour instead of 27,000 x g for 30 minutes to obtain CMF; and

after resuspension of CMF (4.17 ml buffer-KI-EDTA per g of CMF) all fractions were diluted with buffer and/or buffered thyroglobulin or gelatin as indicated in the text under Methods, page .

It had not been noticed in previous experiments that any of the first three modifications had contributed any measurable stability to the enzyme. It is suggested therefore that the additional dilution factor may have acted to stabilize the enzyme to a certain extent and that possibly as much as 25% of the retention of activity may have been due to this added dilution factor alone. This would mean that of the 73% retention of activity noted with thyroglobulin addition only 48% was actually due to the thyroglobulin addition.

V. Evidence for the Existence of Two Thyroid Peroxidases

A. Peroxidase Degradation in Mitochondrial Band

Figures 12, 13 and 14 all indicate that peroxidase activity is located within the mitochondrial band. The activity found in this band was lost after 60 hours as indicated in Fig. 16. The presence of 1% thyroglobulin,

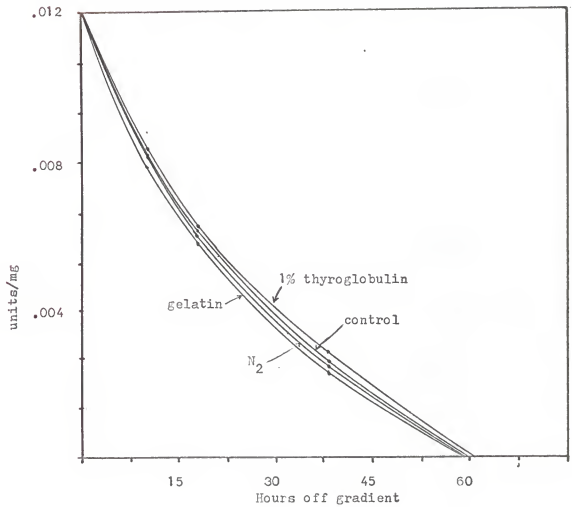

Fig. 16. Peroxidase degradation in mitochondrial band.

1% gelatin and nitrogen atmosphere had no effect at all in maintaining peroxidase activity in the mitochondrial band. It was originally thought possible that peroxidase activity contained in the mitochondrial band might maintain its activity for a longer period of time due to some type of protective action of the relatively high concentration of protein found within the mitochondrial band. The data, however, did not support this theory since gelatin had almost no effect in maintaining activity in either the peroxisomal or mitochondrial band. It should be noted that the activity of peroxidase in the mitochondrial band is exceedingly low--0.012 units/mg when first taken from the gradient (see Fig. 16).

B. Peroxidase Degradation in the Peroxisomal Band

Figure 17 demonstrates the effect which thyroglobulin, gelatin and nitrogen had on the peroxidase activity as contained within the peroxisomal band. Peroxidase activity in this band was maintained in the presence of 1% thyroglobulin whereas gelatin and exposure to N_2 atmosphere had little or no effect in maintainance of activity. Gelatin maintained only 16.2% of the original activity in this band whereas thyroglobulin maintained 72.5% of the original peroxidase activity at the end of 10 hours when all activity had disappeared in the control.

It is therefore proposed on the basis of the foregoing data that there are in fact two different peroxidases in the thyroid and on the basis that peroxisomal peroxidase appeared to maintain activity in the presence of thyroglobulin whereas mitochondrial peroxidase did not respond to this treatment that the peroxisome may very possible be the primary site for iodide oxidation and thereby the biosynthesis of thyroxine.

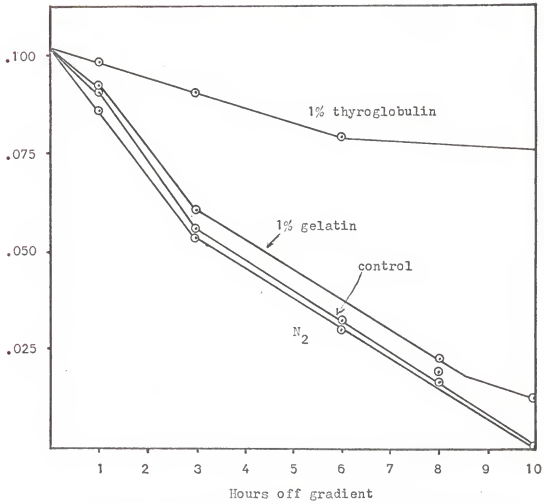

Fig. 17. Peroxidase degradation in peroxisomal band.

VI. Electron Microscopy

A. Subcellular Fractions from Liver

1. Gomori Positive Particles

Figure 18 shows lysosomes taken from the top fraction of a ficoll gradient (described under Methods, page 36). The membrane is still very much intact and protein appeared to be of medium concentration. The major portion of enzymatic activity--acid phosphatase--lay close to the membrane as demonstrated by the presence of PbS stain. No residual bodies are seen within the lysosomal membrane. This, together with the relatively low protein concentration, indicated that these bodies were probably primary lysosomes.

Figure 19, on the other hand, indicated a lysosome in which the acid phosphatase activity appeared much more evenly distributed throughout the particle. Protein concentration is also much higher in this particle than in the others preceeding. This then indicated that the lysosome from the middle band (Fig. 19) had been engaged in the process of phagocytosis and thereby had an endogenous substrate on which to act as opposed to those lysosomes which showed a positive Gomori reaction only close to the membrane. The short incubation time--10 minutes--would allow for a positive reaction of the acid phosphatase contained within the lysosome with the exogenous substrate near the membrane but probably not much further into the particle.

In general, mitochondria were poorly preserved. A characteristically hollow mitochondrial inclusion body is seen just to the right of the lysosome in the center. These bodies are characteristically found in 0.25 M sucrose preparation of "blown" mitochondria (Crofts and Chappell, 1965). All micrographs are of double stained particles.

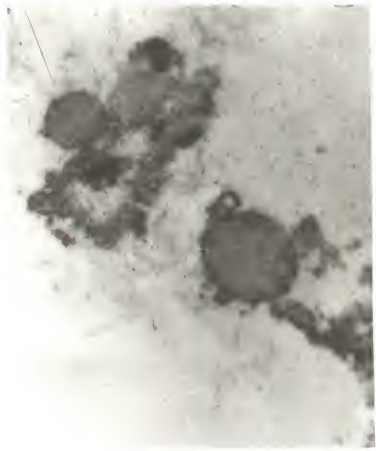

Fig. 18. Lysosomes from rabbit liver stained with Gomori reaction mixture (X 28,487).

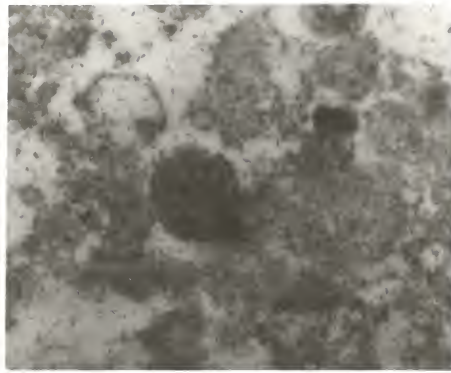

Fig. 19. Gomori-positive lysosomes from middle band in rabbit liver subcellular fraction (X 37,674).

2. Peroxisomes

Figure 20 demonstrates a unit membrane structure taken from the bottom band of the ficoll density gradient. This band exhibited enzyme activity characteristic of peroxisomes. Catalase and uricase were both in strong evidence in this band. The peroxisome pictured in Fig. 20 has a homogeneous concentration of protein characteristic of these particles. A tear in the membrane with consequent spillage of enzyme can be seen. The characteristic core, site of uricase activity, is likewise in evidence.

B. Subcellular Fractions from Thyroid

1. Lysosomes

Figure 21 shows a group of particles which are highly concentrated in protein. These particles were received from the light lysosomal-mitochondrial fraction and were double stained with lead citrate and uranyl acetate. The large lysosone in the upper middle of the picture and that to the lower right of this lysosome are clearly pericanicular as described by Novikoff, et al. (1956). The particle in the lower right of the picture appeared to have been newly engaged in a process of phagocytosis as observed by the presence of a discrete high concentration of protein in one portion of this particle. This could possible be newly ingested thyroglobulin. All lysosomes from this fraction stained very intensely for protein which indicated a highly active state of the particles.

Figure 22 shows lysosomes from the dense lysosomal-mitochondrial fraction. Most of the lysosomes in this fraction (lower portion of micrograph) did not stain as darkly for protein content but rather appeared to contain residual bodies, possible indicating that hydrolysis and discharge of substrates had already occurred. These particles also exhibited a more compact form than that in the lighter density region. This may be explained either

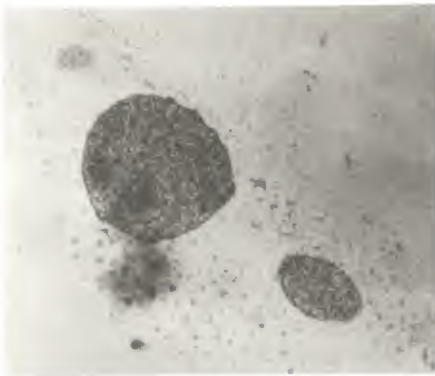

Fig. 20. Peroxisome from rabbit liver (X 30,500).

Fig. 21. Thyroid lysosomes from light density fraction (X 194,628).

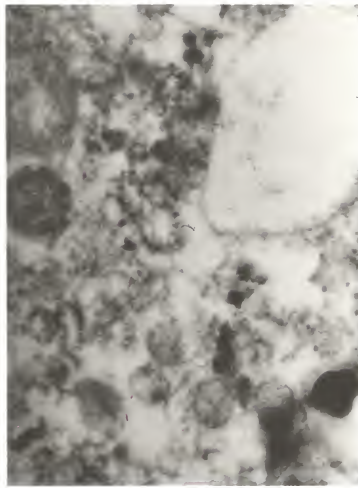

Fig. 22. Thyroid lysosomes from dense lysosomal-mitochondrial fraction (X 75,340).

by some type of osmotic effect from the low density of ficoll in this region or by an effect of phagocytosis by these particles. The upper left of Fig. 22 exhibits a well preserved mitochondrion which could not be demonstrated in liver fractions which were isolated in 0.25 M sucrose.

2. Peroxisomes

Figures 23 and 24 show two well defined particles from the peroxisomal band of the gradient. These particles both have very prominent irregular cores and suggest that hog thyroid peroxisomes may very likely have multiple cores. The lower left hand corner of Fig. 24 shows a region of free cores indicating that some of the peroxisomes were damaged in the preparation process. This helped to account for the vesicles which were scattered throughout the micrograph. Evidently, the peroxisomal membranes fused back together to form vesicles either completely or partially void of proteinaceous material. No explanation can be offered for the aggregation of free cores in this area.

C. Thyroid Slice

An attempt was made to stain for endogenous peroxidase in a thyroid slice. The results, however, were inconclusive as demonstrated by Fig. 25. A stain with benzidine was achieved. However, the stain was so intense as to mask out any particle, if any, in which it was located.

No other staining procedure was used on the section in order to allow for maximum contrast. If one compares the area covered by the stain with that of the peroxisomes in Figures 23 and 24, it appears that the stain is not contained within the peroxisomes.

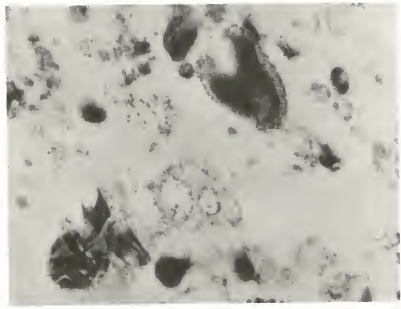

Fig. 23. Thyroid Peroxisome (X 46,360).

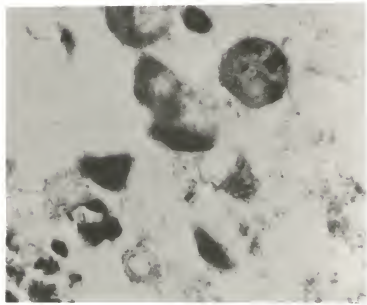

Fig. 24. Thyroid peroxisome and free cores (X 46,360).

Fig. 25. Thyroid slice-benzidine stain (X 37,674).

SUMMARY

Separation of complete mitochondrial fraction from rabbit liver into lysosomes, mitochondria and peroxisomes was achieved using a linear sucrose and non-linear ficoll density gradient. The non-linear ficoll gradient proved to effect a more refined separation of lysosomes and mitochondria and restricted the lysosomes to regions of lower equilibrium density. Neither density gradient employed was successful in completely separating lysosomes and mitochondria due to their similar equilibrium densities.

Catalase, as exhibited by rabbit liver, appeared to have a true dual distribution, one component being found in the mitochondria, the other in the peroxisomes, and it was postulated on this basis that in rabbit liver there is a true structural difference between the two enzymes as contained within the two different subcellular particles.

A separation of the complete mitochondrial fraction of hog thyroid was achieved by means of both linear and non-linear ficoll gradients. The non-linear ficoll gradient was found to be effective in obtaining a refined separation of lysosomes into all three species of density--light, medium and heavy and in forcing a wider separation of lysosomes and mitochondria from peroxisomes.

Enzymatic data and electron microscopic analysis confirmed the existence of peroxidase, catalase and uricase active peroxisomes in a subcellular fraction isolated from thyroid tissue. Peroxidase seemed to be associated with a light mitochondrial fraction and catalase with a heavy mitochondrial fraction. In addition, thyroid peroxidase was seen to be a major constituent of the peroxisomes.

BIBLIOGRAPHY

1. Adams, D. H. and Burgess, E. A., Brit. J. Cancer 11, 310 (1957).

2. Alexander, N. M., Anal. Biochem. 4, 341 (1962).

3. Alexander, N. M., Current Topics in Thyroid Research, ed. by Cassano, C. and Andreoli, M., Academic Press, New York, p. 43 (1965).

4. Anson, M. L., J. Gen. Physiol. 22, 79 (1938).

5. Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P. and de Duve, C., Biochem J. 92, 179 (1964).

6. Baudhuin, P., Beaufay, H. and de Duve, C., J. Cell Ciol. 26, 219 (1965a).

7. Baudhuin, P., Muller, M., Poole, B. and de Duve, C., Biochem. Biophys. Res. Comm. 20, 53 (1965b).

8. Baudhuin, P. and de Duve, C., Physiol. Rev. 42, 323 (1966).

9. Beaufay, H., Bendall, D. S., Baudhuin, P. and de Duve, C., Biochem. J. 73, 623 (1959).

10. Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. and de Duve, C., Biochem J. 92, 184 (1964).

11. Beers, R. F. and Sizer, I. W., J. Biol. Chem. 195, 133 (1952).

12. Cassano, C. and Andreoli, M. ed. Current Topics in Thyroid Research, p. 157 (1965).

13. Cavelieri, R. R. and Searle, G. L., Biochem. J. 102, 25c (1967).

14. Chance, B. and Maehley, A. C., in Methods of Biochemical Analysis Vol I, ed. by Glick, D., Interscience, New York, p. 357 (1954).

15. Cleland, K. W. and Slater, E. C., Biochem. J. 55, 566 (1953).

16. Cooperstein, S. J. and Lazarow, A., J. Biol. Chem. 189, 665 (1951).

17. Coval, M. L. and Taurog, A., Federation Proc. 26, 643 (1967).

18. Crofts, A. R. and Chappell, J. B., Biochem. J. 95, 387 (1965).

19. de Duve, C., Gianetto, R., Applemans, F. and Wattiaux, R., Nature 172, 1143 (1953).

20. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Applemans, R., Biochem. J. 60, 604 (1955).

21. de Duve, C., Beaufay, H., Jacques, P., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R. and Coninck, S., Biochim. Biophys. Acta 40, 186 (1960).

22. deDuve, C., Harvey Lecture Ser. 59, 49 (1965a).

23. de Duve, C., Abstr. Vth Meeting Am. Soc. Cell Biol. 27, 25A (1965b).

24. Degroot, L. J. and Davis, A. M., Endocrinology 70, 492 (1962).

25. Degroot, L. J., Thompson, J. E. and Dunn, A. D., Endocrinology 76, 632 (1965).

26. deReuck, A. V. S. and Cameron, M. P. ed. Lysosomes, Little, Brown and Co., New York, (1963).

27. Eisenburg, G. M., Ind. Eng. Chem. (Anal. Ed.), 15, 327 (1943).

28. Ekholm, R. and Strandberg, U., J. Ultrastruct. Res. 20, 103 (1967).

29. Ekholm, R. and Strandberg, U., J. Ultrastruct. Res. 22, 252 (1968).

30. Feinstein, R. M., Arch. Biochem. Biophys. 79, 399 (1959).

31. Folin, O. and Ciocalteu, V., J. Biol. Chem. 73, 627 (1927).

32. Gomori, G., ed. Microscopic Histochemistry, University of Chicago Press, (1952).

33. Herveg, J. P., Beckers, C. and de Visscher, M., Biochem. J. 100, 540 (1966).

34. Holt, S. J. and Hicks, R. M., J. Biophys. Biochem. Cytol. 11, 31 (1961).

35. Holter, H. and Moller, K. M., Exp. Cell Res. 15, 631 (1958).

36. Hosoya, T. and Ui, N., Nature 192, 659 (1961).

37. Hosoya, T., Kondo, Y., and Ui, N., J. Biochem. 52, 180 (1962).

38. Hosoya, T., J. Biochem. 53, 86 (1963).

39. Hosoya, T. and Morrison, M., Biochem. Biophys. Res. Comm. 20, 27 (1965).

40. Hruban, Z. and Swift, H., Science 146, 1316 (1964).

41. Igo, R. P., Mahoney, C. P. and Mackler, B., J. Biol. Chem. 239, 1893 (1964).

42. Klebanoff, S. J., Yip, C. and Kesseler, D., Biochem. Biophys. Acta 58, 563 (1962).

43. Kunitz, M., J. Gen. Physiol. 33, 349 (1950).

44. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. and de Duve, C., J. Cell Biol. 37, 482 (1968).

45. Lockwood, W. R. and Langston, L. L., Anatomical Rec. 150, 129 (1964).

46. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

47. Lynn, J. A., Amer. J. Clin. Path. 45, 704 (1966).

48. Maloof, F. and Soodak, M., J. Biol. Chem. 239, 1995 (1964).

49. McQuillan, M. T. and Trikojus, V. M., Australian J. Biol. Sci. 6, 671, (1953).

50. Miller, G. L., Anal. Chem. 31, 964 (1959).

51. Miller, F. and Palade, G. E., J. Cell Biol. 23, 519 (1964).

52. Nacklas, M. M., Margulies, S. I. and Seligman, A. M., J. Biol. Chem. 235, 499 (1960).

53. Nadler, N. J., Sarkar, S. K. and Leblond, C. P., Endocrinology 71, 120 (1962).

54. Nadler, N. J., Young, B. A., Leblond, C. P. and Mitmaker, B., Endocrinology 74, 333 (1964).

55. Neil, M. W. and Horner, M. W., Biochem. J. 92, 217 (1964).

56. Nicholson, L. M., Ph.D. Dissertation, Kansas State University, Manhattan, Kansas (1967).

57. Novikoff, A. B., Podber, E., Ryan, J. and Noew, E., J. Histochem. Cytochem. 14, 685 (1953).

58. Novikoff, A. B., Beaufay, H. and de Duve, C., J. Biophys. Biochem. Cytol. 2, 179 (1956).

59. Novikoff, A. B., Ciba Foundation Symposium on Lysosomes, Churchill, London, p. 36 (1963).

60. Nunez, J., Mauchamp, J., Macchia, V. and Roche, J., Biochem. Biophys. Acta 107, 247 (1965).

61. Nunez, J., Mauchamp, J., Jerusalmi, A. and Roche, J., Biochim. Biophys. Acta 145, 127 (1967).

62. Paigen, K., J. Biol. Chem. 206, 945 (1954).

63. Pincus, G., Thimann, K. V. and Astwood, E. B., ed. The Hormones, New York (1964).

64. Porta, E. A., de la Iglesia, F. A. and Hartroft, W. S., J. Histochem. Cytochem. 14, 685 (1966).

65. Rhodin, J., Aktiebolaget Godvil Stockholm. (1954).

66. Rouiller, C. and Bernhard, W., J. Biophys. Biochem Cytol. 2, 355 (1956).

67. Sawant, P. L., Shibko, S., Kumta, U. S. and Tappel, A. L., Biochem. Biophys. Acta 85, 82 (1964).

68. Shibko, S., Pangborn, J. and Tappel, A. L., J. Cell Biol. 25, 479 (1965).

69. Shnitka, T. K., J. Ultrastruct. Res. 16, 598 (1966).

70. Singer, T. P. and Lusty, C. J., Biochem. Biophys. Res. Comm. 2, 276 (1960).

71. Singer, T. P., Kearney, E. B. and Bernath, P., Methods in Enzymology. Vol. V, p. 605 (1962).

72. Singer, T. P. and Kearney, E. B., in McElroy, W. D. and Glass, B., Amino Acid Metabolism, The John Hopkins Press, Baltimore, p. 558 (1955).

73. Straus, W., J. Biophys. Biochem. Cytol. 3, 933 (1957a).

74. Straus, W., J. Biophys. Biochem. Cytol. 3 1037 (1957b).

75. Straus, W. and Oliver, J., J. Exp. Med. 102, 1 (1955).

76. Thomson, J. F. and Klipfel, F. J., Arch. Biochem. Biophys. 70, 224 (1957).

77. Tsukada, T., Mochizuki, Y. and Fujiwara, S., J. Cell Biol. 28, 449, (1966).

78. Vaes, G., Biochem. J. 97, 393 (1965).

79. Vaes, G. and Jacques, P., Biochem. J. 97, 389 (1965).

80. Wattiaux, R., Rutquerts, M. J. and Wattiaux-de Coninck, S., Abstract 6th Intern. Congr. Biochem., New York, p. 669 (1964).

81. Wattiaux, R., Wibo, M. and Baudhuin, P., Ciba Foundation Symposium on Lysosomes. London: Little, Brown & Co. Boston, p. 669 (1963).

82. Wetzel, B. K., Spicer, S. S. and Wollman, S. H., J. Cell Biol. 25, 593 (1965).

83. Whittaker, V. P., Regulation of Metabolic Processes in Mitochondria, Elsevier, Amsterdam, p. 1 (1966).

84. Wollman, S. H., Spicer, S. S. and Burstone, M. S., J. Cell Biol. 21 191 (1964).

STUDIES ON ENZYME DISTRIBUTION IN SUBCELLULAR PARTICLES OF LIVER AND THYROID

by

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ABSTRACT

The vast majority of investigations of subcellular enzyme systems have concentrated on subcellular enzyme systems of rat liver, the result being a tendency to accept all data obtained from this organ as being a model for all other tissues. Little attention has been paid to the possibility that other tissues may exhibit different subcellular properties and enzyme distributions.

The study was therefore undertaken to determine if a species difference did exist and if so how it was manifest in the enzyme distribution as exhibited on a density gradient. To this end, separation of complete mitochondrial fraction from rabbit liver into lysosomes, mitochondria and peroxisomes was achieved using a linear sucrose and non-linear ficoll density gradient. The non-linear ficoll gradient proved to effect a more refined separation of lysosomes and mitochondria and restricted the lysosomes to regions of lower equilibrium density. Neither density gradient employed was successful in completely separating lysosomes and mitochondria due to their similar equilibrium densities.

Catalase, as exhibited by rabbit liver, appeared to have a true dual distribution, one component being found in the mitochondria, the other in the peroxisomes, and it was postulated on this basis that in rabbit liver there is a true structural difference between the two enzymes as contained within the two different subcellular particles.

In dealing with tissues other than liver, a fractionation scheme was set up for the thyroid gland. Of particular interest in working with this gland was the possibility of elucidating something of the action of thyroid peroxidase. Iodination as well as peroxidation properties have recently been ascribed to this enzyme.

Numerous separation and purification schemes have been employed by various authors for this enzyme. These experiments have all pointed to the fact that nothing is known of the exact locus of action of the enzyme. In recent years evidence has suggested that there may actually be more than one peroxidase in the thyroid. This evidence, however, has been based on imperfect separation schemes with little or no knowledge as to which subcellular particles were involved.

A separation of the complete mitochondrial fraction of hog thyroid was achieved by means of both linear and non-linear ficoll gradients. The non-linear ficoll gradient was found to be effective in obtaining a refined separation of lysosomes into all three species of density and in forcing a wider separation of lysosomes and mitochondria from peroxisomes.

Enzymatic data and electron microscopic analysis confirmed the existence of peroxisomes in a subcellular fraction isolated from this tissue. Thyroid peroxidase was seen to be a major constituent of this particle, although, like catalase it was also found to constitute a mitochondrial component.

The dual location of peroxidase in subcellular particles suggested that there might be two different enzymes involved in thyroid metabolism. Inactivation experiments with the peroxidase from these two particles--mitochondria and peroxisomes--provided convincing evidence that this assumption was correct and that the thyroid peroxidase located in the peroxisomes was actually the thyroid peroxidase-iodinase described by Nicholson (1967).